



# ACTA PHYSIOLOGICA SCANDINAVICA

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picomole	nmol, pmol
meter, millimeter, micrometer, nanometer	m, mm, $\mu$ m, nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s <sup>-1</sup> )
newton (force)	N (kg m/s <sup>2</sup> )
pascal (pressure)	Pa (N/m <sup>2</sup> )
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m <sup>2</sup> )

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(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg
(bar)	(1.333 bar)
(millibar)	mbar (100 Pa)
cure	Ci
liter, milliliter, microliter	l, ml, $\mu$ l
degree Celsius	°C

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## A Comparison between Direct and Indirect Measurements of Blood Flow in the Follicular Ovary of the Rabbit

By

PER OLOF JANSSON and GUNNAR SELSTAM

Received 1 July 1974

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### Abstract

JANSSON P O and G SELSTAM *A comparison between direct and indirect measurements of blood flow in the follicular ovary of the rabbit* Acta physiol scand 1975 94 1-7

A comparison was carried out between direct and indirect measurements of blood flow in follicular ovaries of anesthetized laparotomized rabbits. Direct measurements by timed quantitative sampling of ovarian venous blood yielded blood flow values 3 times higher than those found with the use of  $^{15}\text{O}$  radioactive microspheres. However, when simulating the surgical trauma associated with direct measurements, the microsphere technique gave the same high ovarian flow values, indicating that this type of trauma caused ovarian hyperemia. It is concluded that direct measurements of blood flow as performed in the present study are unsuitable for investigations of circulatory events in the ovary. Mechanically induced ovarian vasodilatation is probably a factor which helps to explain the diverging data on blood flow obtained with direct and indirect methods. The radioactive microsphere technique appears to provide a means for reasonably reliable ovarian blood flow measurements in laboratory animals.

---

Conflicting data have been presented in the literature concerning the magnitude of ovarian blood flow. In the sheep, for instance, direct measurements after cannulation of the ovarian vein have yielded blood flow values about ten times higher than some indirect techniques such as krypton clearance and indicator dilution (Setchell 1969, Mattner and Thorburn 1969).

The present investigation was undertaken to analyze some of the possible reasons for the diverging results in earlier flow measurements. Direct measurements of venous outflow were compared with indirect measurements using radioactive microspheres. A study was performed under standardized conditions in anesthetized sexually mature virgin rabbits. The aim of the study was to find a method which permits correct measurements of blood flow in this type of ovaries under physiological conditions.

### Materials and Methods

#### *Animals*

Female albino Swedish Land rabbits, 5-6 months old and weighing  $\approx 3$  kg, were used. They had not previously been mated and their ovaries contained follicles and had a mean weight of  $91 \pm 4$  mg. The animals were deprived of food 12-4 h before the experiment.

TABLE 1. Direct measurements of blood flow of the left ovary in unoperated rabbits. <sup>a</sup> Values are expressed as Mean  $\pm$  S.E.

n	Ovarian weight (mg)	Mean arterial blood pressure (mm Hg)	Blood flow in left ovarian vessels <sup>b</sup> (ml/min)		Ovarian blood flow (ml/100 g/min)
			Before clamping	After clamping	
6	54 $\pm$ 12	95 $\pm$ 6	0.71 $\pm$ 0.07	0.29 $\pm$ 0.04	4.1 $\pm$ 0.7

<sup>a</sup> Connections between ovaries and uterine vessels ligated on the left side.

<sup>b</sup> Measurements performed immediately before and after clamping of ovarian vessels at  $\alpha$  level. Difference in flow rate considered to reflect left ovarian blood flow.

Group no. 1 had the vascular supply to both ovaries intact and the area round the ovaries was not manipulated.

Group no. 2 had the ovario-uterine vascular connections ligated and divided on the left side 10 minutes prior to the measurement. The right ovary was left intact.

Group no. 3 had bilaterally intact vascular supply but the left ovary was gently manipulated for 10 s by squeezing it between thumb and index finger 10 min before the measurements.

Table II summarizes the data from these three groups. There were no statistically significant differences in blood flow rate per unit of weight between the two ovaries of the first group. Neither did the flow rate of the right, untouched ovary of the other two groups differ significantly from those of the first group. Ligation of the ovario-uterine vessels or manipulation of the ovary without ligation of the vessels caused a threefold increase in blood flow as measured by this technique 10 min after the mechanical procedure.

The mean blood flow to the untouched ovaries of the 15 rabbits was 155  $\pm$  12 ml/100 g/5 min (21 ovaries).

The renal blood flow measured in the caudal parts of both kidneys in each rabbit was

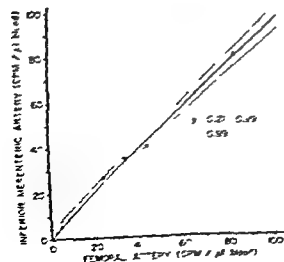


Fig. 2. Correlation between pressure of renal artery and inferior mesenteric artery in rabbits. The correlation coefficients (r) for the three series are: O = 0.97, □ = 0.98, Δ = 0.99. The correlation coefficients for the three series are: O = 0.97, □ = 0.98, Δ = 0.99. The correlation coefficients for the three series are: O = 0.97, □ = 0.98, Δ = 0.99.

TABLE II Blood flow to follicular ovaries and kidneys as measured by  $15 \pm 5 \mu\text{m}^2$  Ytterbium labelled microspheres in anaesthetized laparotomized rabbits. All values are expressed as Mean  $\pm$  S.E.

Group no	n	Experimental model	Mean arterial blood pressure (mm Hg)	Ovarian weight (mg)		Ovarian blood flow (ml/100 g $\times$ min)		Renal blood flow (ml/100 g $\times$ min)	
				Right	Left	Right	Left	Right	Left
1	6	Intact connections between ovarian and uterine vessels on both sides	$86 \pm 6$	$97 \pm 7$	$96 \pm 9$	$160 \pm 23$	$173 \pm 20$	$287 \pm 38$	$279 \pm 36$
2	3	Connections between left ovarian and uterine vessels ligated. Connections on right side intact	$78 \pm 8$	$75 \pm 8$	$74 \pm 8$	$144 \pm 37$	$460 \pm 61^a$	$303 \pm 46$	$305 \pm 44$
3	4	Intact connections between ovarian and uterine vessels on both sides. Left ovary manipulated <sup>b</sup>	$89 \pm 8$	$114 \pm 11$	$108 \pm 8$	$136 \pm 1$	$436 \pm 96^a$	Not measured	Not measured

<sup>a</sup>  $p < 0.05$  compared to the right side<sup>b</sup> Squeezed between thumb and index finger for 10  $\pm$  10 ms before measurement

$295 \pm 20$  ml/100 g  $\times$  min (22 kidneys in 11 rabbits) with no significant differences in flow rate between the left and the right side. Ligation of vessels around the left ovary inevitably involves manipulation also of the adjacent left kidney but as indicated by the data from Group no. 2 (Table II) this procedure did not measurably influence renal blood flow.

### Discussion

Direct measurements of blood flow from an intraabdominal organ with a complex vascular supply inevitably involves laparotomy and mechanical interference with the organ and its vessels. This may result in unphysiological blood flow values. Goding *et al.* (1972) reviewing results from various studies of ovarian blood flow in the sheep by direct venous cannulation suggested that one important source of error in such measurements might be a reduction in ovarian blood flow caused either by an increase in venous pressure or a mechanically induced spasm of the ovarian artery. This view finds support in a recent study carried out in this laboratory of blood flow in the heavily luteinized ovaries of the pseudopregnant rabbit (Janson and Albrecht 1975). In that study direct measurements by venous cannulation resulted in almost 3 times lower values than indirect measurements using radioactive microspheres. In the present study of the follicular ovary the vein was transected eliminating the risk of an increased venous pressure. When this technique was compared to indirect measurements in intact ovaries using radioactive microspheres the former technique resulted in 3 times higher values than the latter. To analyze whether the discrepancy in flow values was

produced by the procedures involved in direct blood sampling, *i.e.* ligation of ovario-uterine blood vessels: a preparation for direct blood flow measurement was simulated and ovarian blood flow was measured in the preparation by the use of microspheres. In this preparation there was good agreement between results from the two techniques: the ovarian blood flow values being three times higher than in intact ovaries. If the ovario-uterine vascular connections were left intact while the ovary was bluntly manipulated, the ovarian blood flow as measured with microspheres was increased to the same level as in the ovaries whose ovario-uterine connections were ligated. These data indicate that even gentle handling of the ovary causes a considerable increase in blood flow. The sensitivity of the follicular ovary to manipulation is a definite drawback when using indirect techniques for flow measurements involving a local application of indicator directly into the ovarian tissue. Such techniques also have other drawbacks when applied to the ovary which is an organ with several compartments with different perfusion rates (for a discussion see Lassen and Larsen 1972).

The present modification of the microsphere technique for blood flow measurement involves no local trauma to the ovaries and the technique can even be performed in conscious animals (Heutze *et al.* 1968). The present study and similar investigations in this laboratory have shown that the intracardiac infusions of microspheres cause no significant changes in blood pressure, cardiac output and heart rate. The spheres have been shown to distribute homogeneously in the aortic blood stream as indicated by the results from the present study and when reaching the ovary they do not shunt to the venous side (Åhrén *et al.* 1974). The number of spheres trapped in the ovaries of the present study permits ovarian blood flow measurements with a precision of 5–10% at a 95% confidence level. Ovarian blood flow can thus be measured using microsphere technique with a reasonable accuracy in a small experimental animal like the rabbit and the technique seems to be suitable for further studies of ovarian blood flow regulation.

The mechanism of the increase in ovarian blood flow evoked by the preparation procedure for direct blood flow measurement is unclear. Damage to vessels and vasomotor nerves may cause the release of vasoactive substances such as adenosine compounds, potassium ions and biogenic amines (Folkow and Neil 1971). Recently a similar mechanically induced vasodilatation has been demonstrated in the small intestine (Biber *et al.* 1971) probably evoked *via* a local nervous reflex and probably involving 5-hydroxy tryptamine as a mediating substance. This substance was also suggested to be involved in acute intestinal vasodilatation in response to gastrointestinal hormones (Biber *et al.* 1974). In the ovary it has been shown that certain gonadotrophic hormones induce acute and marked hyperemia (Ellis 1961; Wurtman 1964) in which both nervous and humoral factors have been suggested to be involved. An investigation of the mechanisms of ovarian hyperemia in response to different stimuli using a reliable technique for blood flow determination is of interest and might possibly give new insight into the mechanism of action of gonadotrophins on the ovary.

This research was sponsored by grants from the Swedish Medical Research Council (B74-03X, 710C), the National Institutes of Health (LSPH (5R01 HD 07507), Göteborgs Läkarsällskap, Creta and Harald Jansson's Foundation and the Faculty of Medicine, University of Göteborg. We are indebted to Professor Kurt Åhrén for valuable suggestions. Skilful technical assistance was given by Miss Kersti Gustafson.

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## The Release of Prostaglandin-Like Substances during Platelet Aggregation and Pulmonary Microembolism

By

J VAAGE and PRISCILLA J PIPER

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### Abstract

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During pulmonary microembolism subsequent to induced platelet aggregation *in vivo* a pressor response is elicited in the lungs due to the release of vasoactive substances. During the perfusion of isolated cat lungs with whole blood platelet aggregation induced by collagen released prostaglandin like substances (PG LS) and a rabbit aorta contracting substance (RCS) as measured by continuous bioassay of the venous effluent. This release occurred in parallel to the pulmonary pressor response. The same amount of PG LS and RCS was released when the lungs were replaced by a blood filter to trap platelet aggregates. Apparently PG LS and RCS are released from platelets during their aggregation. As E and F type PGs are rapidly inactivated in the pulmonary circulation there must either be an equal generation of PGs in the lungs themselves, or rather the release of PGs from platelets must occur distal to the inactivation mechanism without any additional PG release from the lungs. The exact role of PGs in the lung responses to intravascular platelet aggregation is not clear.

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Circulating blood platelets may attain increased adhesiveness and aggregability and they may even aggregate during pathophysiological states (Dhall *et al* 1969 Ljungquist 1973). Such platelets and platelet aggregates will to a large extent be trapped in the pulmonary vascular bed (Ljungquist Bergentz and Lewis 1971). Pulmonary retention of platelets and platelet aggregates has again been claimed to cause lung dysfunction and to be a component in the pathogenesis of acute respiratory distress (Bo and Hognestad 1973 Blaisdell and Schlobohm 1973 Blaisdell 1974). When platelet aggregation is induced *in vivo* a powerful constriction of the smooth muscles in both lung vessels and in airways is elicited. This constriction is probably caused by the release of vasoactive substances from platelets or from the lungs (Rådegran and McAslan 1972, Vaage Bo and Hognestad 1974). Prostaglandins (PGs) may be important in this connection since PGs ( $E_2$  and  $F_2$ ) are released in platelet rich plasma during aggregation (Smith and Willis 1970 Smith *et al* 1973) Piper and Vane (1971) have also demonstrated release of PGs from lungs after various mechanical stimuli including embolism with various non biological particles (0.8-120  $\mu$ m diameter).

In the present work we investigated whether PGs are released in parallel with the pul-

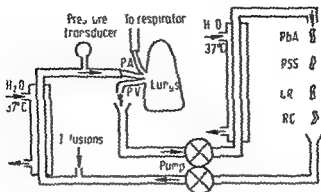


Fig 1 Diagram of the apparatus for perfusion of isolated cat lungs. Ventilated cat lungs were perfused with blood at 37°C. Perfusion pressure was measured in the pulmonary artery (PA). The venous effluent was reoxygenated (37°C) and pumped to superfuse 4 assay tissues: rabbit aorta (RbA), rat stomach strip (RSS), chick rectum (CR) and rat colon (RC). Then the blood was pumped back to the lungs. A blood filter was sometimes used in place of lungs in the circuit.

monary pressor response elicited in the lungs by pulmonary microembolism with induced platelet aggregates. Additionally we wanted to obtain information whether PGs are released from platelets only from lungs only or from both after induced intravascular platelet aggregation. Since rabbit aorta contracting substance (RCS) is released together with PGs we also decided to investigate the release of this substance.

## Methods

The method used was a modification of the blood bathed organ technique (Vane 1964; Piper and Vane 1969). Cats weighing 2.5–4.0 kg were anesthetized with pentobarbitone sodium 30–40 mg/kg given intraperitoneally and then heparinized (500 IU/kg). In each experiment one cat was used as a blood donor and exsanguinated by cardiac puncture. Another cat was initially bled 30 ml from a catheter in the carotid artery to obtain additional blood and then served as lung donor. A tracheotomy was performed and continuous positive pressure ventilation commenced (5 ml/stroke, 22 strokes/min). This ventilation is close to the normal one for cats of this size (Vaage unpublished observations). The end expiratory tracheal pressure was kept at about 2 cm H<sub>2</sub>O by means of a water seal. The chest was rapidly opened, the lungs and the heart removed and the pulmonary artery and the left atrium were cannulated. The isolated lungs were then perfused in the following way. From a reservoir the blood was pumped at 25 ml/min into the pulmonary artery. In all experiments the pulmonary arterial pressure was between 10 and 0 mm Hg. The pulmonary venous outflow pressure was kept constant at  $\pm 2$ –3 cm H<sub>2</sub>O by adjusting the outlet of the left atrial cannula. The blood escaped into a reservoir and was then pumped to superfuse 4 assay tissues. The 4 tissues included rabbit aorta (RbA), rat colon (RC), chicken rectum (CR) and rat stomach strip (RSS). A diagram of the set up is shown in Fig. 1. Changes in smooth muscle tension were measured by Harvard smooth muscle transducers and recorded on a Watanabe 4-channel pen recorder. Before being used for bioassay the tissues were bathed for 1½ h in Krebs solution gassed with 5% CO<sub>2</sub> and 95% O<sub>2</sub> and containing a combination of antagonists that eliminate the actions of histamine, catecholamines, serotonin and acetylcholine (Piper and Vane 1969). These were (in g/ml): hyoscine hydrobromide  $10^{-5}$ , propranolol hydrochloride  $2 \times 10^{-5}$ , metylsergid bitartrate  $\times 10^{-5}$  and mepyramine maleate  $10^{-5}$ . Calibrating doses of PGE<sub>2</sub> and PGF<sub>2</sub> were repeatedly infused directly to the assay tissues. Perfusion pressure was measured by recording the pressure in the pulmonary artery.

## Results

In order to induce platelet aggregation a suspension of collagen fibrils (Bo and Hognestad 1972) was infused into the pulmonary artery (1 ml over 1 min). The observation



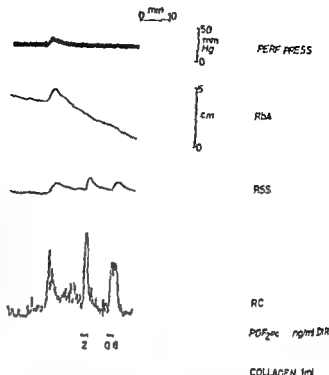


Fig. 2. Pulmonary perfusion pressure and contractions of 3 assay tissues (rabbit aorta (RbA), rat stomach strip (RSS) and rat colon (RC)) subsequent to infusion of collagen into the pulmonary artery. Collagen infusions increase perfusion pressure and release a rabbit aorta contracting substance (RCS) and PG like substance(s) (PG LS). PG LS in this example could be matched by calibrating doses of  $\text{PGF}_{2\alpha}$ .

monary pressor response is a verification that platelet aggregation has been induced. Collagen per se has no effect on the pulmonary circulation (Vaage, Bø and Hognestad 1974). A total of 7 collagen infusions into lungs were performed in 5 expts. Such infusions always caused a rise in the perfusion pressure of 10–20 mm Hg about 1–2 min after the end of each infusion, and all assay tissues always contracted (Fig. 2). The time course of the contraction and the rise in perfusion pressure (Fig. 2) were similar. This contraction was short lasting. The only substances so far found to contract RC, RSS and CR simultaneously in the presence of combined antagonists are PGs of the E and F series (Piper and Vane 1969, 1971). Therefore contraction of this system showed release of some PG like substance(s) (PG LS) during platelet aggregation induced by collagen. The contraction of RbA showed release of a rabbit aorta contracting substance (RCS) which may be a substance intermediate in the biosynthesis of PGs (Gryglewski and Vane 1971), possibly an unstable endoperoxide (Hamberg and Samuelsson 1973).

In each of the 5 expts an additional infusion of collagen was made with the lungs replaced in the circuit by a blood infusion filter with a pore size of  $40\ \mu\text{m}$  (Bentley Polyfilter PF 127 Bentley Lab. Inc.). This was done either initially or at the end of the experiment. The contractions of assay tissues elicited by infusion of collagen into the lungs or filter were compared with those caused by calibrating doses of PGs given directly to the assay tissues. When the filter was used the addition of collagen caused a contraction of the assay tissues which both quantitatively and qualitatively was similar to the contractions seen after collagen infusion into lungs.

The contractions of RC, RSS and CR could usually not be exactly matched by calibrating infusions of either  $\text{PGE}_2$  or  $\text{PGF}_{2\alpha}$  which are released during platelet aggregation (Smith *et al.* 1973) but combinations of these PGs might have caused the contractions in all tissues except RbA. When the active material released was assayed as  $\text{PGE}_2$  or  $\text{PGF}_{2\alpha}$  it appeared that amounts of 1–5 ng/ml were released. Fig. 2 shows one expt. where the contractions could be matched by calibrating doses of  $\text{PGF}_{2\alpha}$ . The concentrations of PGs used for calibration of the assay tissues did not, however, have any effect on the pulmonary perfusion pressure when given i.v. Neither did such infusions cause any contraction of the assay tissues, indicating effective inactivation in the lungs.

As a control the addition of collagen to the assay tissues was tested when they were superfused with Krebs solution instead of blood. No contraction of the assay tissues was then elicited. Neither did histamine nor serotonin doses of 0.5–1  $\mu\text{g/ml}$  given directly to the assay tissues cause any contractions.

### Discussion

One important consequence of induced platelet aggregation in the circulation is smooth muscle constriction in the airways and in the lung vessels supposedly due to humoral factors released from platelets (Rådegran and McAslan 1972; Vaage, Bø and Hognestad 1974; Vaage *et al.* 1974). This pressor response was also observed in the present experiments in spite of lung perfusion with a very low flow. The amount of PG LS detected in blood was small and equivalent concentrations infused did not cause any pulmonary vasoconstriction. However, the concentration locally at the site of the trapped platelet microemboli close to the smooth muscle cells might be far higher.  $\text{PGF}_{2\alpha}$  constricts both bronchial and pulmonary vascular smooth muscles (Ånggård and Bergström 1963; Sweatman and Collier 1968). RCS contracts isolated vascular and bronchial smooth muscle but its action on blood vessels and airways *in vivo* is unknown (Piper and Vane 1969; Piper and Walker 1973). Consequently PGs and RCS might be direct mediators of at least part of the pulmonary pressor response during platelet embolism. This suggestion is supported by the observation that the time course of PG LS release is parallel to the time course of the pulmonary pressor response. Alternatively PGs might modulate and even enhance the actions on smooth muscle of other possible mediators such as for instance 5-hydroxytryptamine as they are known to modulate vascular smooth muscle reactivity (Greenberg *et al.* 1973; Greenberg and Long 1973).

By exchanging the lungs with a filter to trap platelets we hoped to determine quantitatively how the lungs could modify the amount of PG LS and RCS in blood after intravascular platelet aggregation. Theoretically release from the lungs themselves and/or inactivation could take place. PGs infused into lungs are rapidly inactivated in the pulmonary circulation (Ferreira and Vane 1967; Piper, Vane and Wyllie 1970) and PG release from lungs is induced by a variety of mechanical and chemical stimuli (Piper and Vane 1971). However, in the present experiments the same amount of active materials was detected whether lungs or a mechanical filter was in the circuit. This could be achieved by inactivation of PGs released from platelets at the same time as PGs are released from the lung tissue. It seems

unlikely, however, that the lungs would release PGs to exactly compensate for the amount which was inactivated.

Another explanation of our findings is that no PGs are released from lungs. Indeed the platelet aggregates probably have different mechanical properties from the various non biological emboli used in other investigations. PGs released from lungs in these investigations might also be due to secondary changes and not to the emboli per se. PGs released from lungs by anaphylaxis are not available to the inactivation mechanism in the pulmonary circulation (Piper 1974) and PGs released from platelets may also not be inactivated in the pulmonary vascular bed. This could easily happen if PG release occurs downstream to this mechanism. In the present experiments there is no reason to question the capacity of the pulmonary inactivation mechanism, which at least was able to remove PGs in the concentrations detected during release when PGs were infused into the pulmonary artery.

In conclusion, PGs and RCS are released in parallel to the pulmonary pressor response during pulmonary microembolism due to intravascular platelet aggregation. However as the presence of lungs in the circuit did not modify the amount of PGs detected after induced platelet aggregation it seems possible that PGs and RCS are released from platelets only and not from lungs as well. These vasoactive substances might be involved in the vascular and bronchial smooth muscle constriction observed in the lungs after induced intravascular platelet aggregation.

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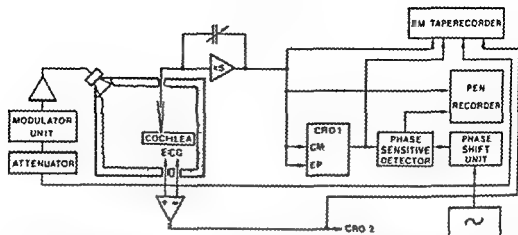


Fig. 1. Experimental arrangement for recordings of the endolymphatic potential (EP) and the cochlear microphonic potential (CM) from the pigeon with a microelectrode. The recorded signals from the cochlea were processed as shown by the block diagram. The stimulus tones were delivered from a loudspeaker placed in the ceiling of a sound attenuating box. The ECG was recorded and displayed on an oscilloscope (CRO 2). For more detailed description see Methods.

was artificially respiration through a silicone rubber tube placed in the trachea (30 strokes/min). By a three-way stopcock the pigeon could be respiration with air or nitrogen.

During the experiment the pigeon was covered by cotton wool to minimize heat loss. The rectal temperature was found to decrease about 1°C/h after administration of Tubocurarin chloride. The total duration of the experiments was usually 2–3 h.

#### *Surgical procedures*

When a satisfactory level of anaesthesia had been achieved an incision was made posterior in the opening of the ear canal. The muscles were carefully dissected away with minimal blood loss until the bone of the skull was uncovered. The wall of the skull was opened by a dental drill (air driven) and the bone trabeculae between the skull and the labyrinth removed with watchmaker's forceps leaving a clear view to the proximal part of the cochlea. The cochlear wall was carefully thinned with a fine burr close to the window region. The drilling was stopped when the connective tissue (peristyle) of the inner wall of scala vestibuli was reached. This tissue was perforated with a sharpened needle.

#### *Anatomy*

The gross anatomy and histology of the avian cochlea have been described by Retzius (1884). A more detailed account of the distribution of the differentiated hair cells along the papilla basilaris and the innervation pattern of the hair cells has been given by Takasaka and Smith (1971).

#### *Electrode*

Highly cleaned glass tubes (Corning, code 7740) were drawn on a horizontal electrode puller. The diameter measured by light microscopy was 0.18–0.2  $\mu\text{m}$ . The tips of the pipettes were filled in distilled water (Zeuthen 1973) and the shaft with 3 M KCl through a hypodermic needle. The pipettes were then placed in a bath and immersed in membrane filtered 3 M KCl (pore size of the filter 0.1  $\mu\text{m}$ ). Before the pipettes were mounted in an electrode holder with a Ag/AgCl electrode the electrolyte of the shaft was replaced by a 0.15 M KCl solution. Electrodes prepared in this way had a resistance of  $10^9$  M $\Omega$  measured in Ring solution and a tip potential of less than 5 mV.

#### *Registration*

The measuring circuit is shown in diagrammatic form in Fig. 1. The pigeon was grounded via a salt bridge (0.15 M KCl in 3 Ag) between undamaged muscles in the neck and an Ag/AgCl electrode. The microelectrode was mounted in a modified micromanipulator by which the electrode could be

vanced in steps of  $0.8 \mu\text{m}$  by a stepmotor (Slo-Syn The Superior Electric Company Bristol Connecticut U S A ) The microelectrode was connected to the input of a preamplifier (Bioelectric, Negative Capacitance Amplifier NF 1) and the output from this stage representing the potential difference between the microelectrode and ground was fed to the input of an oscilloscope (Tektronix 50L A) a potentiometer pen recorder (Servogor) and a FM tape recorder (Philips Analog 7) The output from the preamplifier which also contained an ac signal (CM) when the ear was stimulated with sound was fed into the second channel of the oscilloscope (ac-coupled) After suitable amplification the signal was fed to a Phase Sensitive Detector (Brookdeal 411) and to a tape recorder A sine voltage from a function generator (Wavetek) served as reference signal for the phase sensitive detector The sine voltage was frequency and phase locked to the tone generator (Hewlett Packard 200 CD) which governed the sound stimulus The phase of the reference signal could be varied continuously from 0 to  $360^\circ$  The output of the phase sensitive detector was recorded on the second channel of the potentiometer recorder This output signal represented the amplitude of CM when the phase angle between CM and the reference signal was 0 or  $180^\circ$

#### Sound stimulus

The output of the tone generator (HP 200 CD) was fed to a modulator unit where the pulse duration, on off rise times of the pulse and repetition rate could be selected These signals were fed to a power amplifier which was connected to a loudspeaker placed in the ceiling of a sound attenuating experimental box The voltage to the loudspeaker was calibrated to give a sound pressure level (SPL) of 70 dB (re  $0.0002 \text{ dyn cm}^{-2}$ ) at the site of the pigeon's head When different SPL's were used the voltage to the loudspeaker was attenuated in 5 dB steps The maximal SPL used in these experiments was 95 dB The calibration procedure was performed with a  $1/8$  condenser microphone (Bruel & Kjaer type 4133) connected to a  $1/3$  octave filter (Bruel & Kjaer type 1612) and a microphone amplifier (Bruel & Kjaer type 606) All frequencies used during the experiments were calibrated at regular intervals

#### Electrode resistance

In order to check the integrity of the electrode tip the electrode resistance was measured at regular intervals during the experiments An intermittent triangular voltage was differentiated by a small capacitor connected to the preamplifier input The biphasic rectangular current pulses flowing through the electrode to the ground generated an input voltage to the preamplifier the amplitude of which was proportional to the total resistance to ground (Lettvin *et al* 1958)

#### Analysis of the cochlear microphonics potential (CM)

When the electrode was in ductus cochlearis the stray capacitance of the recording system was maximally neutralized by negative capacitance feed back giving a time constant for the input of the recording system of less than 0.2 msec This procedure increased the noise of the recording system and consequently small amplitudes of CM were buried in noise For analysis of the amplitude of CM during anoxia the signal was fed to a band pass filter (Bruel & Kjaer type 1612) and a microphone amplifier (Bruel & Kjaer type 606) The rms value of the signal from the amplifier was recorded on the potentiometer recorder In two experiments the signal to-noise ratio was increased by breaking the electrode tip Thus it was possible to investigate the wave form of CM

The relative phase variation of CM during anoxia was analyzed using a Phase Sensitive Detector (PSD) The dc voltage from the PSD is proportional to the voltage of the signal times the cosine of the phase angle between the signal and the reference voltage Thus when signal and reference voltages were  $90^\circ$  out of phase the dc signal from the PSD was zero and independent of amplitude being of the signal voltage Changes in the phase angle of CM during anoxia gave a dc signal from the PSD different from zero Since the voltage is dependent upon the change in phase and the actual amplitude of CM the relative phase angle of CM could be calculated The time constant of the PSD was usually set at 300 ms giving a bandwidth of approximately 0.1 Hz (bandwidth (Hz) =  $1/3 \times \text{time constant (s)}$ )

#### Experimental procedure

The microelectrode was placed in the scala vestibuli close to the columella footplate and advanced with a constant step frequency towards the papilla basilaris without visual control of the electrode tip Both the dc potential and the amplitude of the fundamental frequency of CM were recorded upon the potentiometer recorder The electrode was advanced through the cochlea The electrode tip was assumed to be in the ductus cochlearis when the amplitude of CM increased simultaneously with recording of a positive

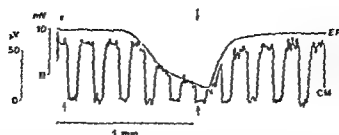


Fig. 1. The effect of transient anoxia on the endolymphatic potential (EP) and the amplitude of CM. The upper trace shows the change in EP during transient anoxia. The lower trace shows the r.m.s. value of the fundamental frequency of CM measured by the microphone amplifier. The ear was stimulated with 4 kHz sound pulses of eight seconds duration at four second intervals. Nitrogen breathing was initiated at the time indicated by the first arrows and terminated 60 s later indicated by the next pair of arrows (the 2 curves are displaced horizontally due to the pen recorder arrangement). The best frequency in this experiment was 4 kHz.

and with only a minor associated phase shift ( $0-30^\circ$ ). Furthermore this positive potential should be sensitive to anoxia.

Since the exact position of the electrode in the ductus cochlearis could not be determined CM frequency response curves were obtained in each animal at 70 dB SPL with the electrode tip in ductus cochlearis. This functional measure of position relative to the papilla basilaris served to compare the results from different pigeons.

The anoxia experiments were performed with the electrode in ductus cochlearis. The pigeons were made anoxic by ventilating with pure nitrogen for 40-90 s. The pigeons were always reventilated with air before bradycardia occurred. During the entire period both EP and CM were recorded. When the amplitude changes of CM at different frequencies were studied successive periods of anoxia were conducted with 5 min intervals between them. In each pigeon the change in CM at one or more frequencies during transient anoxia was investigated at least twice during the course of the experiment. The observed values

CM changes during anoxia were not significantly different indicating that successive periods of anoxia did not affect the demonstrated frequency-dependent change of CM amplitude during anoxia.

## Results

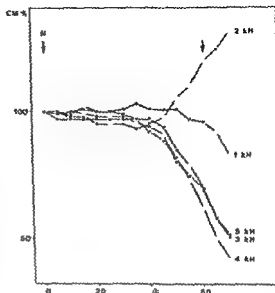
### General pattern of changes

The effect of transient anoxia upon CM at different frequencies at constant sound pressure level (70 dB) was studied in 22 pigeons. In 9 pigeons the effect of transient anoxia upon CM at different frequencies and at varied sound pressure level was investigated. The effect on both the amplitude and the phase angle was examined in all experiments.

Fig. 2 shows an example of the effect of transient anoxia upon EP and the r.m.s. value of CM at 4 kHz. The reduction of CM began 27 s after anoxia was initiated. The corresponding delay for EP was 25 s. The minimum value of CM and EP was reached 8 seconds after readmission of atmospheric air. The recovery of CM and EP followed a more rapid time course than the reduction time course.

In all experiments corresponding values of EP and CM during anoxia were obtained. Since EP is known to be extremely sensitive to anoxia (Konishi, Butler and Fernandez 1961) the duration of anoxia in each experiment was determined by the time to reduce EP by 10-15 mV. This procedure gave a relatively constant change of the electrical potential gradient across the apical pole of the hair cells. The anoxia period varied between 40 and 90 s, but in all experiments with transient anoxia the change in EP was reversible.

Fig. 3 An example of the effect of transient anoxia upon the amplitude of CM at different frequencies but at constant sound pressure level (70 dB). The amplitude of CM is expressed as per cent of the initial value before nitrogen ventilation. Only the amplitude changes of CM during the anoxic period are shown but CM at all frequencies used returned to the initial level after air was readministered. The frequencies used are indicated at the end of the respective curves.



In each experiment the electrode tip was kept in position in the ductus cochlearis. At this position the effect of transient anoxia upon CM at different frequencies and EP was examined. An example of the effect of transient anoxia upon CM at different frequencies is shown in Fig. 3. The amplitude of CM in per cent of the value before anoxia is plotted as a function of time (seconds) during the anoxic period. The last point indicated for CM at all frequencies coincides with the minimum value of EP. EP in these experiments was reduced by 11 mV and the time period from the beginning of reduction to the beginning of recovery ( $\Delta t$ ) was  $43.6 \pm 2.3$  s. CM at 3, 4 and 5 kHz were found to be markedly reduced during the anoxic period and the time courses followed by CM at 3, 4 and 5 kHz were almost identical. In contrast to the behaviour at these frequencies CM at 2 kHz increased during anoxia. After initiation of anoxia the amplitude of CM at 2 kHz was slightly reduced (3–7%). At the time when the amplitude reduction of CM at higher frequencies was accelerated CM at 2 kHz increased and continued to do so during the anoxia period. The last point indicated represents the maximum value and the amplitude returned to the initial level after a slight undershoot of 10%. CM at 1 kHz was less affected by anoxia. The time interval between initiation of anoxia and the reduction in amplitude of CM was larger than at other frequencies and the total reduction in amplitude was only 18% of the initial level.

The CM frequency response curve (tuning curve) for a given position of the electrode tip in ductus cochlearis is shown in Fig. 4. The amplitude of CM in  $\mu V \text{ r.m.s.}$  is plotted as a function of the frequency of the sound stimulus at constant sound pressure level (70 dB). The effect of transient anoxia is a change in the frequency response curve in such a way that the CM tuning curve becomes more flat. In experiments where the low frequency slope of the frequency response curve was less steep than in the example shown the effect of transient anoxia was a change in the best frequency (the frequency at which CM had maximum amplitude) towards a lower frequency. The CM frequency response curve in Fig. 4



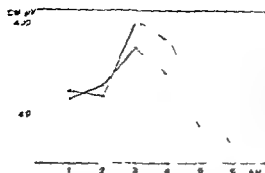


Fig. 4. The effect of anoxia on the CM frequency-response curve. The amplitude of CM is plotted as a function of the frequency in a semi-logarithmic scale. The amplitude of CM before anoxia is indicated by filled circles and the amplitude of CM after 3 a. or 70 s of anoxia (see Fig. 3) is indicated by open circles.

not corrected for the middle-ear transfer function. However, since the pigeon was immobilized with tubocurarine this function remains unaltered during anoxia, and the change in tuning characteristics of CFI is caused by anoxia alone.

#### Amplitude changes of CM

The results of 115 experiments from 22 pigeons are shown in Table 1. The results are divided into 2 groups depending upon the best frequency of the preparation as determined from the CM frequency-response curve obtained at 70 dB SPL.

Table 1 shows the calculated mean relative amplitude of CFI at different frequencies after a period of anoxia. The amplitude of CM was measured at the time when the corresponding EP reached its maximum value, and the change in amplitude is expressed in per cent of the initial value.

The results show that when 3 kHz was the best frequency the average amplitude reduction of CFI was largest at 3, 4 and 5 kHz. CFI at frequencies less than 3 kHz were less reduced in amplitude. CFI at 2 kHz in this group was either enlarged or slightly reduced in amplitude during transient anoxia. An initial enlargement in amplitude of CM during transient anoxia was found in most experiments at this frequency, but in only 16% of the experiments the amplitude continued to increase during the anoxia period.

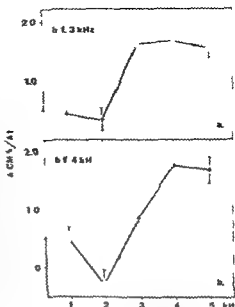
When 4 kHz was the best frequency the mean reduction in amplitude was largest for CFI at 4 and 5 kHz. CFI at 3 kHz was less reduced in this group and sometimes showed an amplitude increase followed by a slight reduction. A conspicuous enlargement in amplitude of CFI at 2 kHz was found in 40% of the experiments; in the rest the amplitude was un-

TABLE 1. The relative amplitude of CM at different frequencies during transient anoxia. The results from 22 pigeons are divided into 2 groups depending upon the best frequency of each preparation. Values are S.E. values for each group (see text).

CM (dB)	Best frequency 3 kHz		Best frequency 4 kHz	
	CM	S.E.	CM	S.E.
CM (1)	10	-4.2 (1)	1	1.2 (1)
CM (2)	11	9.8 (1)	1	9.5 (1)
CM (3)	3	-2.9 (2)	1	4.0 (1)
CM (4)	2	-6.2 (1)	1	11.0 (1)
CM (5)	3	5.3 (1)	1	1.0

Fig. 5 The sensitivity of CM to transient anoxia, expressed as the maximal change in CM in percent of the initial level divided by the corresponding time period in seconds when the ear was anoxic as judged by the reduction in EP ( $\Delta\text{CM}\%/\Delta t$ ) is plotted as a function of the stimulus frequency. The sound pressure level was kept constant at 70 dB. Each point represents a mean value and the numbers of observations are shown in Table I. The standard errors of means are indicated by vertical bars.

Fig. 5 a shows the relation between the sensitivity of CM to transient anoxia and the sound frequency when 3 kHz was the best frequency (maximum amplitude of CM at this frequency). Fig. 5 b shows the relation between the sensitivity of CM and the frequency when 4 kHz was the best frequency.



affected or slightly reduced during transient anoxia. CM at 1 kHz was like the first group showing only slight reduction in amplitude or no amplitude change at all during the test period.

#### *Sensitivity to anoxia*

There is a total reduction of CM at all frequencies in prolonged anoxia (2–4 min). The amplitude change of CM is a function of both the time after initiation of anoxia and the frequency of the sound stimulus. Therefore a better estimate of the frequency-dependent effect of anoxia on CM than the one given in Table I is needed. Such an estimate may be obtained by dividing the relative amplitude change of CM by the time period of anoxia, as judged by the behaviour of EP. This time period ( $\Delta t$ ) was in each experiment taken to be the time between the start of the decrease of EP and the start of the increase in EP after air was readministered (see Fig. 2).

In Fig. 5 a and b the mean sensitivity of CM to anoxia, ( $\Delta\text{CM}\%/\Delta t$ ) is plotted as a function of the frequency of the sound stimulus. The sensitivity of CM at 3, 4 and 5 kHz was higher than at 1 and 2 kHz when 3 kHz was the best frequency. When the best frequency of the preparation was 4 kHz the pattern was different. The sensitivity of CM at 4 and 5 kHz was larger than at 1, 2 and 3 kHz. The mean sensitivity of CM at 4 kHz was found to be significantly different from the mean sensitivity of CM at 3 kHz ( $P < 0.001$ ).

Comparison between the results of Fig. 5 a and 5 b shows that the mean sensitivities of CM at 1, 4 and 5 kHz to anoxia were alike. At 2 and 3 kHz the sensitivity of CM to anoxia was lower when the best frequency was 4 kHz than when 3 kHz was the best frequency. The difference in sensitivity to anoxia at these frequencies was found to be significant ( $P < 0.001$ ). Thus the sensitivity of CM to transient anoxia was found to be dependent upon the frequency as well as the best frequency of the preparation. CM at frequencies equal to

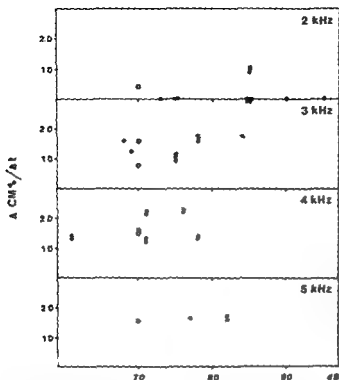


Fig. 6. The sensitivity ( $\Delta CM / \Delta t$ ) of CM to transient anoxia is plotted as a function of the sound pressure level (dB). The frequency of the sound stimulus is indicated. Each point represents one experiment. Open circles represent values of the sensitivity of CM taken from Fig. 5.

and higher than the best frequency was more sensitive to transient anoxia than CM at frequencies lower than the best frequency. CM at frequencies 1 kHz lower than the best frequency often showed a negative sensitivity (corresponding to an increase in amplitude) during transient anoxia.

#### *Effects of intensity differences*

The above described results were obtained at constant sound pressure level (70 dB). Thus at this point it is uncertain whether the observed differential effect of anoxia is a frequency dependent effect or an effect of the difference in absolute amplitude of CM at the used frequencies.

In a separate series of experiments the influence of varied sound pressure level upon the CM sensitivity to anoxia was examined. The sensitivity of CM to anoxia in each experiment was calculated and plotted as a function of the sound pressure level (Fig. 6) for various frequencies of the sound stimulus.

From the distributions of observations for CM at 3, 4 and 5 kHz, the sensitivity of CM to anoxia seems to be unrelated to the sound pressure level. For each frequency the regression line for the observations was calculated. None of the slopes of the lines was significantly different from zero. In experiments where CM at 2 kHz was enlarged in amplitude during anoxia at low SPL, elevation of SPL was found to reduce the enlargement or even cause a minor reduction. The tendency observed in many experiments at frequencies different from 2 kHz, that elevation of SPL reduced the sensitivity could not be shown to be significant either.

TABLE 31 The difference between the time periods for reduction in amplitude (CM) and potential (EP) during transient anoxia at different sound frequencies. Mean values  $\pm$  S.E. and number of experiments

CM (kHz)	(CM-EP) $\pm$ S.E. seconds	n
CM (1)	$8 \pm 5.0$	13
CM (2)	$30 \pm 3.5$	1
CM (3)	$\pm 0.9$	--
CM (4)	$7 \pm 4.4$	13
CM (5)	$18 \pm 1.7$	1
CM (6)	$\pm 0.9$	13
CM (7)	$2 \pm 0.9$	11

The frequency in the bracket is the best frequency

#### The relation between the time courses of CM and EP

The time course of the changes in CM was compared to the corresponding changes in EP during transient anoxia. The time period from the beginning of nitrogen breathing to the first perceptible reduction in CM and EP was recorded (cf. Fig. 2) and the difference was calculated. Table 31 shows the results.

The reductions in CM at 1 and 2 kHz were markedly delayed relative to that in EP. The delay at 3 kHz was not significantly different from zero when 3 kHz was the best frequency but CM at 3 kHz was delayed relative to EP when 4 kHz was the best frequency. The values for CM at 4 and 5 kHz were not significantly different from zero.

The changes in amplitude of CM and in EP were examined at different times during the anoxia period and correlation coefficients between the relative changes in corresponding values of CM and EP were calculated. When the frequency was equal to and higher than the best frequency very high correlation coefficients were found ranging between 0.91 and 0.99 in 26 experiments.

#### Changes in waveform of CM

The above given description of amplitude changes of CM during transient anoxia is restricted to the r.m.s. value of the fundamental frequency of the electrical signal from the cochlea. The influence of anoxia upon the waveform of CM was examined less systematically. In two experiments the electrode tip was broken so that larger signal to noise ratios were obtained thereby allowing for visual examination of the waveform.

From the visual examination the waveform of CM was not changed during transient anoxia. During prolonged anoxia the amplitude of CM at 2 kHz was reduced and the waveform of CM was markedly distorted. This is in contrast to the change in CM at 3 kHz where a distortion of the waveform could not be observed. In these experiments 3 kHz was the best frequency. The distortion of CM at 2 kHz during prolonged anoxia is shown in Fig. 7. The effect of anoxia is seen to be complex. Besides a change in waveform anoxia caused a change in the phase angle of CM relative to the initial phase (see below).

#### The relative phase angle of CM

Anoxia is expected to affect only the mechanisms responsible for EP and eventually the hair cell potential. The differential effect of anoxia upon the amplitude of CM indicates

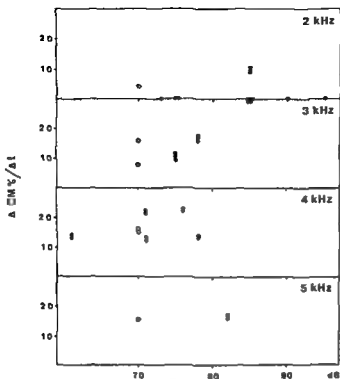


Fig. 6 The sensitivity ( $\Delta CM / \Delta t$ ) of CM to transient anoxia is plotted as a function of the sound pressure level (dB). The frequency of the sound stimulus is indicated. Each point represents one experiment. Open circles represent values of the sensitivity of CM taken from Fig. 5.

and higher than the best frequency was more sensitive to transient anoxia than CM at frequencies lower than the best frequency. CM at frequencies 1 kHz lower than the best frequency often showed a negative sensitivity (corresponding to an increase in amplitude) during transient anoxia.

#### *Effects of intensity differences*

The above described results were obtained at constant sound pressure level (70 dB). Thus at this point it is uncertain whether the observed differential effect of anoxia is a frequency dependent effect or an effect of the difference in absolute amplitude of CM at the used frequencies.

In a separate series of experiments the influence of varied sound pressure level upon the CM sensitivity to anoxia was examined. The sensitivity of CM to anoxia in each experiment was calculated and plotted as a function of the sound pressure level (Fig. 6) for various frequencies of the sound stimulus.

From the distributions of observations for CM at 3, 4 and 5 kHz, the sensitivity of CM to anoxia seems to be unrelated to the sound pressure level. For each frequency the regression line for the observations was calculated. None of the slopes of the lines was significantly different from zero. In experiments where CM at 2 kHz was enlarged in amplitude during anoxia at low SPL, elevation of SPL was found to reduce the enlargement or even cause a minor reduction. The tendency observed in many experiments at frequencies different from 2 kHz, that elevation of SPL reduced the sensitivity could not be shown to be significant either.

The maximum value of the phase shift calculated (Fig. 8 b) corresponds to the value obtained from direct reading on the oscilloscope (Fig. 8 c)

Changes of the phase angle of CM during transient anoxia were investigated in the anoxia experiments described above. The changes in phase of CM were found to be a complicated function of stimulus frequency and the sound intensity. The largest changes in phase were found around the best frequency.

### Discussion

The results of this study show that the effect of transient anoxia on the amplitude changes of CM at constant sound pressure level is dependent upon the frequency of the sound stimulus. When the sound pressure level and thereby the amplitude of CM was changed the differential effect of transient anoxia remained unaltered. This fact excludes the possibility that the demonstrated differential effect of anoxia upon the amplitude changes of CM is due to the difference in amplitude of CM at different frequencies when the sound pressure level is constant. Thus it can be concluded that transient anoxia produces a frequency-dependent change in the amplitude of CM.

As a relative measure of the position of the electrode tip in ductus cochlearis the best frequency was determined from the CM frequency response curve obtained at constant sound pressure level. The best frequency has been shown to change with the position of the electrode up along the basilar membrane (Honrubia and Ward 1963). The best frequency in all experiments in this study was either 3 or 4 kHz, indicating that the electrode tip was located mainly in two positions in the ductus cochlearis.

The division of the results into two groups depending upon the best frequency of each preparation gives two anoxia sensitivity curves (sensitivity of CM as a function of the frequency Fig. 5). They both show that CM at frequencies equal to and higher than the best frequency is more sensitive to transient anoxia than CM at frequencies lower than the best frequency. The findings that CM at 3 kHz sometimes behaves like low frequency CM and sometimes like high frequency CM appears to be related to the best frequency of the preparation. When CM at 3 kHz is the best frequency the sensitivity of CM at this frequency to transient anoxia is high. The relation between the sensitivity of CM to transient anoxia and the best frequency suggests that the effect of anoxia is a positional one. The difficulties in gaining access to more distal parts of the ductus cochlearis with a microelectrode prevented further verification of this suggested positional effect of anoxia.

Using differential electrodes placed along the basilar membrane in guinea pig Dallos (1973 b) recently demonstrated that the 3 dB cut-off frequency of the CM frequency response curve at low SPL (40 dB) corresponded to the frequency which produced maximal vibration of the basilar membrane at that location. CM frequency response curves recorded with a single electrode in ductus cochlearis of the pigeon at low SPL resemble CM frequency response curves from the guinea pig recorded with a differential electrode technique. They are similar with respect to non linearity of the best frequency and the high and low frequency slope (Jørgensen in prep). This similarity in CM frequency response curves from the guinea pig and the pigeon suggests that CM recorded with a single electrode in d

chleairis does reflect the displacement pattern of the basilar membrane better than previously believed

A consequence of the frequency dependent change of CM is that transient anoxia produces changes in the CM frequency response curve (cf Fig 4). This curve becomes flattened and the best frequency may be shifted towards a lower frequency. The shift is a consequence of the difference in sensitivity of CM to transient anoxia but may be emphasized by an increase in amplitude of CM at 1 kHz lower than the best frequency.

Although the mechanism for the frequency dependent change in amplitude of CM during transient anoxia is not known, the finding that the sensitivity of CM at different frequencies is related to the best frequency rather than the numerical value of the stimulus frequency indicates that the effect of transient anoxia is positional. The change in the CM frequency response curve produced by transient anoxia suggests that changes in the mechanical tuning of the basilar membrane take place.

Transient anoxia was formerly believed to influence the endolymphatic potential only but recent investigations of the vibration pattern of the basilar membrane of the squirrel monkey during death showed that the tuning of the basilar membrane is physiologically vulnerable (Rhode 1973). A decrease in amplitude of vibration by a factor of 10 over a 10 min period immediately after death was observed. The first and most marked changes occurred for frequencies near and greater than the frequency which produced maximal vibration of the area of the basilar membrane under observation. This region of the basilar membrane attained a lower frequency as the best frequency but the non linear behaviour of this region (Rhode 1971) disappeared. These changes in the tuning characteristics of the basilar membrane during death were accompanied by changes in the phase of the vibration relative to the stapes. The changes in pattern of vibration of the basilar membrane may indicate that both an increase in damping and a decrease in stiffness of the basilar membrane take place during death.

Recently Evans (1973) and Klinke and Evans (1973) demonstrated that hypoxia and metabolic inhibitors caused changes in the frequency threshold curves of single cochlear fibres in the guinea pig. The low threshold sharply tuned curves became broadly tuned high threshold curves. In some cases the characteristic frequency was shifted towards a lower frequency when metabolic inhibitors were administered. These findings were interpreted as a loss of a second filter subsequent to that of the basilar membrane. This filter has been proposed to account for the difference in tuning between the basilar membrane and the single cochlear fibre. The finding however may also indicate that hypoxia and metabolic inhibitors cause changes in the vibration pattern of the cochlear partition.

When the changes in vibration pattern of the basilar membrane of the squirrel monkey during death are compared with the frequency-dependent changes in amplitude of CM in the pigeon during transient anoxia the striking qualitative resemblance is noted. Although the interpretation of the CM amplitude changes is dependent upon more precise knowledge of the quantitative relation between extracellular recorded CM and the corresponding vibration of the basilar membrane underneath the electrode tip, the results give evidence that transient anoxia causes similar changes in the vibration pattern of the basilar membrane in the pigeon ear. This would mean that the amplitude changes of CM during anoxia were

caused by the combined effect of changes in the endolymphatic potential and changes in the mechanical vibration pattern of the cochlear partition. The demonstrated changes in the relative phase angle of CM during transient anoxia with major changes around the best frequency are in agreement with this concept.

In search for the mechanism responsible for the changes in the vibration pattern of the cochlear partition during anoxia, the close coupling in time between the reduction in the endolymphatic potential and the amplitude reduction of CM at frequencies equal to and higher than the best frequency may be of significance. Since only a part of the amplitude change of CM can be accounted for by the change in the electrical potential gradient (Davis model), the remaining change of CM may be caused by the suggested changes in pattern of vibration which then must follow a time course similar to that of EP. The findings of Klinke and Evans (1974) may support this notion since metabolic inhibitors most likely have the largest effect on the endolymphatic potential. Changes in the hydrostatic pressure in ductus cochlearis may be the relevant change since Tonndorf (1957) demonstrated that an increase in volume of the endolymphatic space in a cochlear model caused a reduction in the amplitude of the basilar membrane vibration. The reduction was found to be frequency dependent with largest amplitude reduction in the distal end of the cochlea (lower frequencies). Furthermore a shift in locus of maximum displacement amplitude for a particular frequency towards the proximal end was observed.

Recently Necker (1970) reported that transient anoxia produces complex changes in microphonic potential in pigeon, starling and sparrow. CM was elicited by acoustic clicks (rarefaction and condensation) and recorded with electrodes in the perilymphatic space. The results have been interpreted in terms of the hair cell transducer mechanism and do not appear to offer an explanation for the phenomenon investigated in this study.

In conclusion it has been shown that transient anoxia causes a frequency-dependent change of the amplitude of CM. The results suggest this effect to be a positional one such that CM at frequencies equal to and higher than the best frequency is more sensitive to anoxia than CM at frequencies lower than the best frequency. CM at 1 kHz lower than the best frequency often increases in amplitude during transient anoxia. It has been proposed that the frequency dependent change in CM amplitude is a consequence of changes in the vibration mode of the basilar membrane caused by anoxia. Thus the amplitude changes of CM during anoxia are caused by the combined effect of changes in EP (the electrical potential gradient across the apical pole of the hair cells) and changes in the pattern of vibration in the cochlear partition. The observation that changes in the amplitude of CM are associated with changes in the relative phase angle of CM supports this hypothesis.

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## Transcapillary Fluid Movements in Sympathectomized Intestine and Skin during Hemorrhagic Hypotension

By

JOHANNESE JÄRHULT and PER OLOF GRANDE

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### Abstract

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Net transcapillary fluid exchange in skin (paw) and small intestine was observed during a 90 min period of hemorrhagic hypotension at 50 mm Hg in the cat. Reflex fluid transfer was prevented by regional sympathectomy and  $\alpha$  adrenergic blockade. Early in hemorrhage fluid absorption from the extravascular space occurred in both tissues, apparently caused by osmosis. The process was thus coordinated in time with a positive arterio-venous osmolar difference, in turn caused by a marked arterial hyperosmolality. Experimental arterial hyperosmolality of similar magnitude created by i.v. infusion of hypertonic glucose in non bled animals led to transcapillary fluid absorption in both intestine and skin and at rates similar to those in bleeding. Regional hypotension per se caused no fluid absorption. Later in hemorrhage (> 30 min) plasma fluid moved into the extravascular space both in skin and intestine, apparently due to a gradual increase of capillary hydrostatic pressure. It is concluded that the arterial hyperosmolality during bleeding can cause transcapillary fluid absorption in intestinal and skin tissues, as previously shown for skeletal muscle (Järehult 1973). The hemodynamic significance of this process for plasma volume regulation in hemorrhage is however much greater in skeletal muscle than in intestine and skin mainly due to the much larger total mass of the muscle tissue.

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During hemorrhagic hypovolemia extravascular fluid from skeletal muscle is mobilized into the blood stream. Thus transcapillary fluid absorption is caused partly by an adrenergic reflex decrease of the capillary hydrostatic pressure (e.g. Öberg 1964) and partly by an osmotic mechanism related to arterial hyperosmolality in turn created by glucose release from the liver (Järehult 1973). The latter study indicated that the osmotic mechanism was responsible for about 50 per cent of the total extravascular fluid absorption from cat skeletal muscle during hemorrhagic hypotension at 50 mm Hg. In the present investigation an attempt was made to analyse whether such an osmotic fluid transfer can occur in tissues other than skeletal muscle, i.e. intestine and skin.

### Methods

The study was performed on cats anesthetized with x-bio aloxe (50 mg/kg) initially supplemented a small dose of pentobarbital sodium (10-15 mg). The animals were deprived of food for 16 h before the experiment.

**Intestinal preparation** In 10 cats without obvious signs of intestinal infection an intestinal preparation was made according to that described by Folkow *et al* (1963). Briefly a segment of the jejunum (weighing 15–20 g) was isolated together with its lymph node and the remainder of the small and large intestines removed. The regional autonomic nerves were ligated and cut. A short arterial shunt circuit was inserted in the superior mesenteric artery. The venous outflow from the segment and its lymph node was measured with an optical drop recorder and returned to the animal via the jugular vein. To record changes in tissue volume the intestinal segment was enclosed in a perspex temperature controlled (38 °C) plethysmograph filled with Tyrode's solution.

**Skin preparation** The hind paw was chosen for the study of the circulatory events in a cutaneous vascular bed (12 cats). The pads (with abundant arterio-venous anastomoses) were excluded from the circulation by tight ligatures. Hence the reactions observed can be considered to have occurred mainly in nutritive skin vessels. The skin of the calf was dissected free from the knee down to the ankle joint. All superficial and deep veins were ligated except for the great saphenous vein which was cannulated and the venous outflow diverted to the jugular vein via an optical drop recorder. The paw was placed in a perspex plethysmograph filled with water using the calf skin flap for proximal closure. The temperature was kept at 34 °C (= neutral temperature). In 2 expts the circulatory events in the paw were studied during regional hypotension. For this purpose a short shunt circuit was inserted between the femoral and popliteal artery, all other arterial vessels between the thigh and the calf being ligated. Regional arterial pressure was then monitored from a T-tube in the shunt and could be adjusted to desired levels by a screw clamp. The weight of the different tissues in the paw was determined after each experiment. On the average skin was found to comprise 26 per cent, tendons, fat and muscle 41 per cent and bone 53 per cent (in agreement with Öberg 1964).

**General experimental procedure** After heparinization the right carotid artery was cannulated and connected to a siliconized pressure bottle to permit bleeding. Mean arterial blood pressure was monitored from the left femoral artery and measured with a Statham P23 AC transducer. The regional  $\alpha$ -adrenoceptors in the intestine and skin were blocked with a close arterial injection of phenoxylbenzamine (1–3 mg). To avoid systemic effects of this drug, the venous outflow from the region was collected for 3 min after the injection, discarded and substituted by the same amount of dextran. The effectiveness of the  $\alpha$ -adrenoceptor blockade was checked by close arterial injections of noradrenaline.

Changes of regional vascular resistance were expressed as per cent of control resistance before bleeding. Changes of tissue volume were continuously recorded with a Grass FT 10C transducer connected to the plethysmograph. With this technique a rapid change of tissue volume after bleeding represents the capacitance response and later slower changes represent transcapillary fluid movements (for details see Grande *et al* 1974). Plasma osmolality was analysed with thermistor cryoscopy (Adv. Instruments Inc.). Spread of data is given as S.E.

**Protocol** The animals breathed spontaneously and were permitted to recover for 30 min after the completion of the surgery. Venous outflow pressure in the intestine and the skin was adjusted so as to accomplish an isovolumetric state (transcapillary fluid equilibrium) in the control period. Late in this period blood samples were withdrawn from the carotid artery and from the superior mesenteric or great saphenous vein for determinations of control plasma osmolality. The animal was then bled rapidly (< 5 min) to mean arterial pressure of 50 mm Hg, a level maintained throughout the experiment by means of the pressure bottle. Samples for arterial and venous plasma osmolality determinations were taken 5, 10, 15, 30, 40, 60 and 90 min after the start of the bleeding.

## Results

### Intestine

The control data before hemorrhage in the sympathectomized  $\alpha$ -blocked small intestine (8 expts) were as follows: Arterial inflow pressure  $104 \pm 6$  mm Hg, venous outflow pressure  $7 \pm 3$  mm Hg, blood flow  $33 \pm 4$  ml/min, 100 g regional resistance  $3.0 \pm 0.7$  mm Hg/(ml/min  $\times$  100 g), arterial plasma osmolality  $320 \pm 3$  and venous (superior mesenteric) plasma osmolality  $322 \pm 2$  mOsm/kg H<sub>2</sub>O.

Hemorrhage to an arterial pressure of 50 mm Hg caused a marked gradual decrease of the intestinal vascular resistance (Fig. 1, panel A); after 30 min of hypotension it was 17

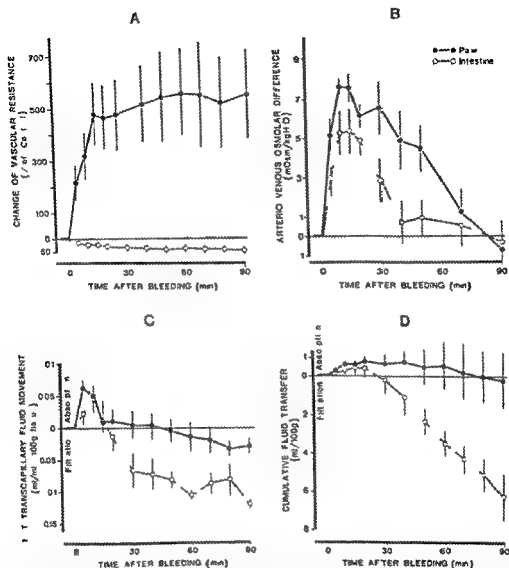


Fig. 1 Circulatory venis in the sympathectomized or blocked paw (●—● 8 expts) and small intestine (○—○ 8 expts) during hemorrhagic hypotension at 50 mm Hg in the cat. Panel A shows the change of vascular resistance; panel B the arterio-venous osmolar difference; panel C the net transcapillary fluid movement; and panel D the cumulative capillary fluid transfer. Mean values  $\pm$  S.E. are given.

duced to about 50% of the control value implying that blood flow then had returned to the prehemorrhagic level. Arterial plasma osmolality increased by about 20 mOsm/kg H<sub>2</sub>O above control after 20–30 min of arterial hypotension in accordance with previous findings (Jarhult 1973). A moderate positive arterio-venous osmolar difference was noted in the early (< 30 min) phase of hemorrhage (Fig. 1 panel B) indicating that a transcapillary osmolar gradient was present in this period (see Lundvall 1972).

The concomitant net transcapillary fluid movement depicted in Fig 1 panel C Absorption of extravascular fluid to the blood stream occurred in the initial period of hemorrhagic hypotension and at a maximal rate of  $0.045 \pm 0.021$  ml/min  $\times 100$  g tissue The absorption process then declined and there was a reversal to net fluid filtration which after 30 min of hemorrhage and later occurred at rates of  $0.07-0.12$  ml/min  $\times 100$  g The fluid absorption was present at a time when the positive arterio-venous osmolar difference was most pronounced suggesting that it was an osmotic process related to the hemorrhagic hyperosmolality This hypothesis was supported by experiments on non bled cats (2 expts) which were merely exposed to an arterial hyperosmolality induced by slow intravenous infusions ( $n=5$ ) of hypertonic glucose solutions Such arterial hyperosmolality ( $+15$  to  $25$  mOsm/kg  $H_2O$ ) regularly evoked a transcapillary fluid absorption in the  $\alpha$  blocked small intestine which amounted to  $0.05-0.1$  ml/min  $\times 100$  g tissue

The cumulative capillary fluid transfer in the intestine during hemorrhage depicted in Fig 1 panel D A fluid gain to the blood stream occurred during the initial 25 min (maximal gain  $\sim 0.5$  ml/100 g tissue) after which a fluid loss occurred This loss averaged 6 ml/100 g of intestine after 90 min of hemorrhage —The initial passive decrease of regional blood volume (capacitance response) was  $0.66 \pm 0.05$  ml/100 g tissue

#### Paw

Hemorrhage experiments were made on 8 cats In the control period arterial inflow pressure averaged  $109 \pm 5$  mm Hg venous outflow pressure  $5 \pm 2$  mm Hg blood flow  $12.1 \pm 3.6$  ml/min 100 g and regional vascular resistance  $8.7 \pm 2.3$  mm Hg/(ml/min  $\times 100$  g) Arterial and venous (great saphenous) plasma osmolality was  $318 \pm 2$  and  $321 \pm 3$  mOsm/kg  $H_2O$  respectively

In contrast to the resistance response in the intestine vascular resistance in the sympathectomized  $\alpha$  blocked paw increased markedly after hemorrhage and stabilized at a level about 500% above control after 15 min of hypotension (Fig 1 A) Regional blood flow thus decreased to very low values being about  $0.8$  ml/min  $\times 100$  g after 30 min This large increase of flow resistance was probably caused by local passive factors (e.g. elastin recoil cell aggregation etc) Evidence for such an interpretation was obtained from regional hypotension experiments (see below) and further by the finding that blood flow quickly increased to the control level and roughly in parallel with the increase of the perfusion gradient when the low perfusion pressure in hemorrhage was restored to control by transfusion

Plasma osmolality rose much more rapidly in the arterial than in the cutaneous blood which led to a clearcut positive arterio-venous osmolar gradient in the paw the first hour of bleeding (Fig 1 panel B) A net transcapillary fluid absorption in the initial ( $<40$  min) period of hemorrhage (panel C) i.e. at the time when the arterio-venous osmolar difference was most pronounced This suggests that the observed fluid absorption was an osmotic phenomenon an opinion further strengthened by the following A similar increase of arterial plasma osmolality evoked by the administration of hypertonic glucose intravenously to non bled animals caused a fluid absorption of  $0.04$  ml/100 g in the paw Late in hemorrhage a slight fluid filtration occurred (panel C)

tive fluid transfer in the paw is depicted in Fig 1 panel D. At 40 min the total fluid gain from the paw to the circulation averaged  $0.8 \pm 0.5$  ml/100 g tissue; it then slowly declined due to the transcapillary filtration loss and after about 80 min of hypotension the net fluid gain was zero. The initial passive capacitance response was  $1.1 \pm 0.14$  ml/100 g in these experiments.

Transcapillary fluid movements in the sympathectomized paw were also studied in non bled animals ( $n=2$ ) in response to regional arterial hypotension (50 mm Hg) maintained during 90 min. This local hypotension caused no capillary fluid transfer during the first 5–10 min but later a net fluid filtration ( $0.02$ – $0.06$  ml/min  $\times$  100 g). Vascular resistance in the paw increased markedly also in these experiments  $\approx$  200–400%.

The figures for fluid transfer given above are calculated for 100 g soft paw tissue; bone excluded.

### Discussion

This study has shown that a transcapillary fluid absorption is present in intestinal and cutaneous tissues in early stages of hemorrhage. For the following reasons this absorption of extravascular fluid is believed to be caused at least mainly by an osmotic process evoked by the hemorrhagic arterial hyperosmolality: (a) The regions were sympathectomized and the  $\alpha$  adrenoceptors blocked which excluded fluid absorption by  $\alpha$  adrenergic resetting of the pre/postcapillary resistance ratio (cf Öberg 1964). (b) The absorption took place in a period when the arterio-venous osmolar difference (reflecting the transcapillary osmolar gradient) was clearly positive (Fig 1 panels B and C). (c) When the arterial osmolality was increased in non bled cats to the same level as in hemorrhage by means of a slow intra-venous infusion of hypertonic glucose a fluid absorption of a similar magnitude to that during bleeding was observed in the intestine and paw. (d) Regional arterial hypotension ( $\approx$  50 mm Hg) caused no fluid absorption in the paw nor does this occur in the small intestine as reported by others (Haglund and Lundgren 1972).

It may be argued that some non adrenergic vasoconstrictor substance (e.g. angiotensin and/or vasopressin cf Bond, Manley and Green 1967; McNeill *et al.* 1970) could have caused a capillary pressure fall by an increase of the pre/postcapillary resistance ratio hence contributing to the fluid absorption. Such an effect was apparently not present in the gut since hemorrhage led to an intestinal vascular resistance decline, a finding in agreement with Haglund and Lundgren (1973). In the paw however a vascular resistance increase did occur but this seemed to be caused mainly by passive factors as a similar increase was evoked by regional hypotension *per se*; the resistance increase in the latter case in fact was associated with a net fluid filtration indicating that in relative terms it was mainly confined to the postcapillary resistance vessels. It cannot be entirely excluded that the intravascular instrumentation in the present experiments to some extent was responsible for the marked vascular resistance increase in the skin during hemorrhage and regional hypotension. If so such interference has led to some underestimation of the magnitude of the osmotic transcapillary fluid absorption in this tissue since the vascular resistance increase *per se* during hypotension caused a net fluid filtration thus opposing the osmotic absorption.

Absorption of extravascular fluid was however, observed only during the first 20–40 min of hemorrhagic hypotension. Later on fluid was lost from the blood stream into the extravascular space, an effect particularly pronounced in the intestine (0.1 ml/min  $\times$  100 g tissue). This filtration most likely can be ascribed to an increased capillary hydrostatic pressure ( $P_c$ ) both in the intestine and the paw. An increase of  $P_c$  in the intestine thus is compatible with the observed decline of vascular resistance, but mention should be made that this increase of  $P_c$  apparently amounted to less than 1 mm Hg. This can be deduced by taking into consideration the known large intestinal capillary filtration coefficient during bleeding (0.1–0.15 ml/min  $\times$  100g  $\times$  mm Hg, see Haglund and Lundgren 1973). The rise of  $P_c$  in the paw, as mentioned, may be due to passive factors (e.g. elastic recoil, increased viscosity, cell aggregation, etc.) which are known to preferentially affect the postcapillary resistance vessels (Eriksson and Lisander 1972).

In the present experiments the intestine and paw were sympathectomized and the  $\alpha$  adrenoceptors blocked to facilitate the revelation of a possible osmotic absorption effect. There are some earlier studies during hemorrhage on transcapillary fluid exchange in the intestine and paw with intact adrenergic innervation. In the intact intestine most studies have revealed a net fluid filtration, especially in late stages of hemorrhage (e.g. Johnson and Selkurt 1958, Glenert and Pedersen 1967, Cook, Wilson and Taylor 1971, Haglund 1973), but some authors have reported a net fluid absorption (e.g. Johnson and Selkurt 1958, Marty and Zweifel 1971). During small bleedings of short duration ( $\sim$  10 min) Öberg (1964) found no effect whatsoever on the transcapillary fluid exchange, a phenomenon which he attributed to a virtually unchanged pre- to postcapillary resistance ratio. Such bleedings, however, seems to cause small or negligible increases of plasma osmolality (Järhult and Lundvall, to be published) and hence no osmotic fluid absorption can be evoked. In the intact cat paw (Öberg 1964) and dog foreleg (Schwinghamer *et al.* 1970, Greka *et al.* 1971) hemorrhage has been shown to cause a fluid absorption, which was attributed to adrenergic resetting of the pre- to postcapillary resistance ratio.

Fluid transfer from the extravascular space of skeletal muscle into the blood stream is an important mechanism for plasma volume restoration in hemorrhage. This effect is caused by a reflex adrenergic as well as a hyperglycemic osmotic mechanism, each about equally important from a quantitative point of view (Järhult 1973). The present study suggests that hemorrhagic hyperglycemia causes an osmotic transcapillary fluid absorption also in the intestine and the skin. Although this response thus is qualitatively similar in the 3 vascular beds, the rate of transcapillary fluid absorption per unit tissue weight is higher in muscle than in skin and intestine, and the effect is longer maintained in the former tissue during bleeding (Järhult 1973). These differences may at least partly be explained by the known dissimilarities in capillary ultrastructure. The capillaries in skeletal muscle and skin are characterized by a continuous endothelium, whereas they are fenestrated in the intestinal mucosa. It seems likely that the reflection coefficient in fenestrated capillaries is lower than in such with continuous endothelium and that therefore the transcapillary osmotic fluid absorption is less pronounced. It should be stressed that skeletal muscle is the most important tissue for the plasma volume restoration in bleeding, simply due to its huge tissue mass. In fact it may be calculated that the transcapillary osmotic fluid withdrawal from all

intestine or all skin tissues would comprise less than 5 per cent of the concomitant osmotic fluid absorption from all skeletal muscles

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## Adrenergic and Cholinergic Innervation of the Rat Urinary Bladder

By

PER ALM and MATS ELMÉR

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### Abstract

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The autonomic innervation of the rat urinary bladder was studied using histochemical methods and nerve stimulations. A sparse adrenergic innervation of the detrusor muscle was found. It was supposed to originate from long adrenergic neurones. The trigonum area had a rich supply of adrenergic fibres, probably derived from short adrenergic neurones. A uniformly rich supply of acetylcholine-esterase (AChE)-positive nerves was found in the whole bladder. Postganglionic sympathetic denervation caused no detectable change of or AChE-positive nerves in the bladder, while parasympathetic decentralization or denervation induced a total disappearance of adrenergic fibres. The AChE-positive nerves were appreciably reduced in number after parasympathetic decentralization and not detectable after postganglionic denervation. Neither adrenergic nor AChE-positive ganglion cells could be demonstrated in the bladder wall. Electrical stimulation of the hypogastric nerves or the pelvic nerves distal to the pelvic ganglia elicited contraction of the detrusor muscle. The responses were not affected by hexamethonium, dihydroergotamine or propranolol but were slightly reduced by guanethidine, reduced to about 40% by atropine and potentiated by eserine. Stimulation of the pelvic nerve proximal to the pelvic ganglion was partially blocked by hexamethonium. It is concluded that the urinary bladder of the rat is supplied by postganglionic adrenergic fibres mainly via the pelvic nerves and only to a lesser extent via the hypogastric nerves. Probably cholinergic fibres pass to the bladder mainly via the pelvic nerves but also via the hypogastric nerves, having their cellbodies outside the bladder wall, partly proximal to the pelvic ganglia.

**Key word.** Urinary bladder fluorescence histochemistry; acetylcholine-esterase; denervation; nerve stimulation.

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Physiological and pharmacological evidence indicates that the urinary bladder of the rat in contrast to other species is devoid of intramural ganglion cells (Vanoy 1965; Carpenter and Rand 1966; Elmer 1973). These observations have been supported in morphological investigations by some authors (Carpenter and Rubin 1967; Chesher 1967) while others have found intramural cholinergic ganglion cells in the rat bladder (El-Badawi and Schenk 1966).

Stimulation of the hypogastric nerve causes contraction of the bladder in the dog (Gianuzzi 1863), in the cat and monkey (Sherrington 1892), in the dog, cat and rabbit (Langley and Anderson 1894) and in the guinea pig (Montecazza and Naimzada 1967; Vanoy 1965). Vanoy found no effect of hypogastric nerve stimulation in the rat. In contrast to hypogastric nerve

stimulation injection of the sympathetic transmitter noradrenaline causes relaxation of the bladder in the dog (Dhasmana *et al* 1970) in the cat and the rabbit (Sigg and Sigg 1964 Edvardsen and Setckleiv 1968) and in the rat (Elmer 1974). Sigg and Sigg (1964) proposed that a cholinergic mechanism is involved in sympathetic nerve bladder transmission as postulated by Burn and Rand (1959) for other postganglionic sympathetic neuroeffector systems.

In view of these results the present study was performed to get more information concerning the anatomical origin and distribution of adrenergic and acetylcholinesterase (AChE)-positive nerves of the rat urinary bladder using histochemical methods. Furthermore the effects of electrical stimulation of the hypogastric and pelvic nerves on the urinary bladder before and after the administration of sympathetic and parasympathetic antagonists were studied.

# Methods

168 male albino rats of the Sprague Dawley strain weighing about 400 g were used. The histochemical investigations were performed in non-denervated bladders and in bladders denervated 10-14 days earlier. To make a postganglionic sympathetic denervation the hypogastric nerves were cut distal to the hypogastric ganglia below the bifurcation of the aorta. Parasympathetic decentralization was achieved by the sectioning of the pelvic nerves proximal to the pelvic plexa, which in the male rat form distinct ganglia located on the lateral surface of the prostate gland (Langworthy 1965). In order to make a postganglionic denervation both pelvic ganglia were extirpated (this procedure also interrupts the sympathetic fibres of the hypogastric nerves which pass through the pelvic ganglia). The operations were performed aseptically in ether anaesthesia. In the rats with divided parasympathetic nerves the bladder had to be emptied daily by manual pressure in ether anaesthesia. Despite this treatment these bladders always contained some residual urine and often weighed over 3 times more than controls when examined about 2 weeks after the nerves had been cut. To avoid bacterial infection the animals were given a sulfonamide (Sulfuno® Nordmark Werke 20 mg daily s.c.).

The animals were killed by a blow in the neck whereupon the urinary bladders were rapidly dissected out and according to Wakade and Kirpekar (1972) arbitrarily divided into neck, body and apical portion of the bladder. Thereupon the tissue pieces were rapidly frozen in liquid isopentane cooled by liquid nitrogen.

For the histochemical demonstration of adrenergic nerves pieces of tissue were processed for the method of Falck and Hillarp (Falck *et al* 1962; Björklund *et al* 1972). Serial sectioning was performed from each specimen. In all the experimental groups 3 animals were given Niamid® (Pfizer Ltd 100 mg/kg s.p. 3 h before death) and L DOPA (L 3,4-dihydroxyphenylalanine Sigma Chem. Corp. 40 mg/kg i.v. 1/2 h before death) in order to increase the intensity of fluorescence of the adrenergic nerves (Falck and Hillarp 1965).

Reserpine (Serpasil® CIBA 0.05 mg/kg s.c. 16 h before death) and 6-hydroxydopamine (0 mg/kg i.v. 4 h before death) were tested as to their effects on the adrenergic nerve fluorescence in the bladder compared to heart atria, submaxillary gland, pancreas, vas deferens and seminal vesicle. All tissue pieces were processed for fluorescence histochemistry as described above.

In three rats pieces of tissue were taken from the hypogastric nerves and the pelvic nerves proximally and distally in the places where these nerves were cut in the denervation operations. The specimens obtained were processed for the method of Falck and Hillarp. To avoid a reduction in the fluorescence intensity of the muscular adrenergic bladder nerves (see below) the modifications of Björklund and Falck (1968) were used in the paraffin embedding step and when mounting the sections.

For the demonstration of acetylcholinesterase (AChE) activity the copper diethanol method of Koelle and Friedenwald (1949) as modified by Holmstedt (1957) was used. Cryostatate sections (10 µm) were incubated for 16 h in diethanol in the presence of Mpafox® (bis-methoxypropylammonium dihydrogen phosphate) for the inhibition of nonspecific cholinesterase (Holmstedt 1957; Holmstedt and Sjogquist 1961) or BW 34 C 111 (1,5-bis-4-allyldimethylammoniumphenyl)pentan-3-one dihydrochloride (Wellcome Res. Lab. Beckenham, England) a specific inhibitor of AChE (Bayliss and Tudrak 1956). Eosin was used as counterstain. Mounting was performed in Entellan® (Merck).

For the stimulation experiments 55 rats were anesthetized with chloralose (100 mg/kg) given through a cannula in a femoral vein after induction with ether. The bladder was exposed and the ureters were ligated. A glass cannula was inserted into the bladder through an incision in the urethra. The bladder was filled with 0.5 ml of physiological saline and the pressure developed by the detrusor muscle was recorded by means of a transducer and a polygraph. The resting pressure was about 10 mm Hg in all experiments.

The hypogastric nerves were cut distal to the hypogastric ganglia below the bifurcation of the aorta and the distal ends were stimulated electrically using a bipolar electrode. The left pelvic nerve was stimulated after section proximal or distal to the pelvic ganglion. In order to avoid stimulating the sympathetic fibres from the hypogastric nerves which pass through the pelvic ganglia the hypogastric nerves were cut 10 days before the pelvic nerve stimulations allowing the sympathetic fibres to degenerate. A Grass stimulator giving rectangular pulses with a duration of 2 ms, a frequency of 0.1–0.5 Hz and of supramaximal voltage (5–10 V) was used.

For statistical evaluation of the data Student's *t* test for paired samples was used. The 0.05 level of probability was accepted as significant.

*Drugs.* The substances used in the stimulation experiments were: eserine sulphate, hexamethonium bromide, dihydroergotamine methanesulphonate, phenylephrine hydrochloride, propranolol hydrochloride, guanethidine bisulphate, atropine sulphate, Hoechst 9980 (piperidino-ethyl-diphenylacetamide) and prilocain chloride (Citaneal<sup>®</sup>, Astra, Sweden). The drugs were injected through the cannula in the femoral vein except prilocain which was applied locally on the nerve distal to the site of stimulation.

## Results

### *Histochemical findings*

*Adrenergic nerves.* In non-denervated animals fine varicose nerve terminals very sparsely occurred in the smooth muscular layers throughout the whole bladder except the trigonum area (see below) mostly running between the bundles of smooth muscle cells in their longitudinal direction, sometimes running across the muscle cells connecting longitudinally running terminals thus forming a very slender plexus around the muscular fibres (Fig. 1A). However, in the trigonum area the supply of adrenergic nerve terminals of the smooth muscular layers was very rich, clearly exceeding that of other parts of the bladder. Furthermore, in the trigonum area the varicosities of the adrenergic nerve terminals were clearly more coarse than in the other bladder parts. Fine varicose adrenergic nerves also appeared around some of the blood vessels in the submucosa, muscular and adventitia layers. Despite serial sectioning, no adrenergic ganglion cells could be demonstrated within the rat bladder wall. On the other hand, clusters of ganglion cells could frequently be found outside the bladder wall in the connective tissue surrounding the base of the bladder. In the muscular layers clusters of small intensely fluorescent cells (SIF-cells) could sometimes be found. Treatment with *n*-alamide and L-DOPA did not appear to increase the number of adrenergic nerves. In the specimens from the hypogastric and pelvic nerves bundles of nerves with a specific greenish fluorescence were found.

After pretreatment with reserpine or 6-hydroxydopamine no adrenergic nerve fluorescence was seen in the apical and body portion of the bladder, nor could any fluorescence be found in heart, atria, submaxillary gland and pancreas. However, in the trigonum area of the neck portion of the bladder and in the seminal vesicle and vas deferens the dense adrenergic innervation of the smooth muscular layers was appreciably unchanged. Moreover, some of the blood vessels (smaller arteries and arterioles) in the submucosa of the trigonum area displayed no adrenergic nerve fluorescence.

Postganglionic sympathetic denervation did not appear to influence the supply of either



Fig. 1 A Muscular layers of rat bladder wall. Sparse distribution of adrenergic nerve terminals compared to AChE positive nerves (see Fig. 1 B) Fluorescence micrograph  $\times 140$

Fig. 1 B Section through rat bladder wall. Rich distribution of AChE positive nerves are seen except in the most apical part which consists of the mucosa layer. The large dark spots are AChE positive nerve trunks. Light micrograph  $\times 88$

vascular or muscular adrenergic nerves in any bladder region whether the animals were pretreated with nialamide and L-DOPA or not. After parasympathetic decentralization or postganglionic denervation no adrenergic nerves were detected even if the animals were pretreated with nialamide and L-DOPA.

**AChE positive nerves** The number of nerves demonstrating AChE activity very distinctly exceeded that of nerves demonstrating adrenergic fluorescence (Fig. 1 B). Along the bundles of smooth muscle cells in the muscular layers AChE positive nerve fibres regularly appeared originating from larger trunks of AChE positive nerve fibres lying most basally in the muscular layers. No apparent differences as to the number and distribution of AChE positive nerves could be found between the different regional parts of the bladder. In addition in some of the vessels there could be observed small AChE positive branches ending in the adventitia. No AChE positive ganglion cells could be demonstrated within the bladder wall despite serial sectioning.

Postganglionic sympathetic denervation did not seemingly influence the supply of AChE positive nerve fibres. Parasympathetic decentralization produced an appreciable reduction in the number of AChE positive nerve fibres. However, the AChE positive nerve fibres were still present in the bladder wall.

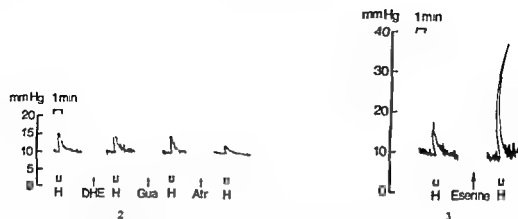


Fig. 2. Pressure responses of the rat urinary bladder to electrical stimulation of the hypogastric nerves (H) at 15 Hz. DHE dihydroergotamine 2 mg/kg Gua guanethidine 1 mg/kg Atr atropine 1 mg/kg.

Fig. 3. Pressure responses of the rat urinary bladder to electrical stimulation of the hypogastric nerves (H) at 15 Hz before and after the injection of eserine 0.2 mg/kg.

nerve fibres could be seen the number apparently somewhat greater in the neck than in the other bladder portions. After postganglionic denervation there was an almost complete disappearance of AChE positive nerve fibres.

#### stimulation experiments

**Sympathetic stimulation** Electrical stimulation of the hypogastric nerves caused contraction of the detrusor muscle. Maximal pressure response was obtained using a stimulation frequency of 15 Hz, which gave an increase in the bladder pressure of  $5.6 \pm 0.7$  mm Hg (mean  $\pm$  S.E. n = 27).

The responses to stimulation of the hypogastric nerves were not affected by previous injection of hexamethonium 10 mg/kg, dihydroergotamine 2 mg/kg, phenoxybenzamine 5 mg/kg or propranolol 2 mg/kg. As shown in the table, guanethidine 1 mg/kg reduced the response by about 25%. Atropine in a dose of 0.1 mg/kg reduced the response by about 40% and after the injection of 1 mg/kg the reduction was about 60%. The parasympatholytic agent Hoechst 9950 (Schaumann and Lindner 1951) had the same effect. The pressure responses to electrical stimulation of the hypogastric nerves before and after the injection of dihydroergotamine, guanethidine and atropine are shown in Fig. 2.

After injection of 0.2–0.4 mg/kg of eserine the response to stimulation of the hypogastric nerves at 15 Hz was increased about 3 times (Fig. 3).

**Parasympathetic stimulation** Electrical stimulation of one of the pelvic nerves proximal or distal to the pelvic ganglion caused contraction of the detrusor muscle. Maximal pressure response was obtained with a stimulation frequency of 15 Hz, increasing the intravesical pressure by  $36.4 \pm 2.5$  mm Hg (n = 28).

The responses to stimulation of the pelvic nerve distal to the pelvic ganglion were not affected by previous injection of hexamethonium 10 mg/kg, dihydroergotamine 2 mg/kg or propranolol 4 mg/kg. Guanethidine 1 mg/kg caused a reduction of the response of

TABLE 1 Pressure responses in mm Hg in the rat urinary bladder to electrical stimulation at 15 Hz of the hypogastric or pelvic nerves before and after the injection of guanethidine 1 mg/kg, or atropine 1 mg/kg. Values are mean  $\pm$  S.E. n = number of rats \* = significance

	Guanethidine		n	p	Atropine		n	p
	Before	After			Before	After		
Hypogastric n	16 $\pm$ 0.4	14 $\pm$ 0.3	7	<0.05	38 $\pm$ 0.6	15 $\pm$ 0.3	8	<0.01
Pelvic n	26.4 $\pm$ 3.8	24.2 $\pm$ 3.7	12	<0.001	29.6 $\pm$ 3.9	12.0 $\pm$ 1.5	13	<0.001

about 8%. Atropine reduced the response by about 60% in a dose of 0.1 mg/kg and the injection of 1 mg/kg did not cause any further reduction (Table 1). When both guanethidine and atropine were given to the same rat the effect of each drug was the same independently of which drug was given first. When the pelvic nerve was stimulated proximal to the pelvic ganglion hexamethonium reduced the response by 50–75%, and the reduction was bigger the more proximal to the ganglion the nerve was stimulated.

Eserine increased the response to stimulation of the pelvic nerve distal to the pelvic ganglion by about 50% in the doses indicated above.

After application of the local anesthetic agent prilocain on the hypogastric or pelvic nerves no response to electrical stimulation of the nerves was obtained, while direct stimulation on the bladder wall still caused a contraction.

### Discussion

In agreement with earlier findings the smooth muscular layers of the rat urinary bladder has a very sparse supply of adrenergic nerves except in the trigonum area where it is much richer (cf. Hamberger and Norberg 1965a; El Badawi and Schenk 1966; Owman and Sjöberg 1972; Wakade and Kirpekar 1972; Sundin and Dahlstrom 1973). Furthermore there is a rich supply of AChE positive nerves in all parts of the bladder very clearly exceeding that of adrenergic nerves (cf. El Badawi and Schenk 1966).

The finding that the responses to electrical stimulation of the hypogastric and pelvic nerves were partially blocked by guanethidine as well as by atropine might also suggest the presence of both adrenergic and cholinergic fibres. A reduction of the response to pelvic nerve stimulation after atropine in the rat has been described earlier (Vanov 1965). An atropine sensitive portion of the response to transmural stimulation of the urinary bladder *in vitro* was found by Chesher (1970) in the rat but not in the cat and guinea pig.

Parasympathetic decentralization and total postganglionic denervation produced a complete disappearance of adrenergic nerves but seemingly no effect was achieved at postganglionic sympathetic denervation. This probably means that the rat detrusor muscle is mainly supplied by adrenergic nerves *via* sacral nerve trunks and not *via* the hypogastric nerves. This could be in line with the findings in the present work demonstrating bundles of adrenergic nerve fibres running in the pelvic nerves. It is also in accordance with previous findings in cats (Hamberger and Norberg 1965b; Sundin and Dahlstrom 1973) but is

trary to findings in guinea pigs (Walade and Karpekar 1972). Furthermore the total disappearance of adrenergic nerves after parasympathetic decentralization and total postganglionic denervation in connexion with the seeming absence of adrenergic ganglion cells within the bladder wall suggest that the adrenergic nerves of the rat bladder wall are peripheral postganglionic fibres (cf. El Badawi and Schenk 1966).

Because of anatomical and functional differences the neurones of the peripheral adrenergic nerve system can be divided into long and short adrenergic neurones (see review by Owman *et al.* 1974). Long adrenergic neurones have a short preganglionic and a long postganglionic fibre. Furthermore the ganglion cells are localized far away from the organ to be innervated. In the short adrenergic neurones the situation is the reverse. In view of this the adrenergic nerve terminals in heart, submaxillary gland and pancreas are supposed to originate from long adrenergic neurones and the adrenergic nerve terminals to seminal vesicle and vas deferens are supposed to originate from short adrenergic neurones (Sjöstrand 1965, Owman *et al.* 1974). In small doses reserpine and 6-hydroxydopamine are known to deplete the transmitter fluorescence of nerve terminals of long adrenergic neurones but not (or to a much slower velocity) in nerve terminals of short adrenergic neurones (Owman and Sjöberg 1967, Malmfors and Sachs 1968, Sjöstrand and Swedin 1968, Jane *et al.* 1970, Jonsson and Sachs 1970). Consequently from the present findings it can be supposed that the adrenergic innervation of the muscular layers and of some of the blood vessels in the trigonum area originates from short adrenergic neurones as no appreciable effect on the adrenergic nerve fluorescence could be demonstrated by reserpine or 6-hydroxydopamine and nor could any effects of these drugs be found in the seminal vesicle or vas deferens. On the other hand fine varicose adrenergic nerve terminals of the smooth muscular layers in the other parts might originate from long adrenergic neurones as no adrenergic nerve fluorescence was simultaneously seen in these bladder parts (nor in the heart, atria, submaxillary gland or pancreas) after treatment with reserpine or 6-hydroxydopamine.

Despite serial sectioning only SIF-cells and no conventional adrenergic ganglion cells were found intramurally in the bladder wall. This verifies earlier studies on the rat bladder wall (El Badawi and Schenk 1966). Although SIF-cells have been found in a lot of various organs their physiological role is still unknown.

The response to stimulation of the hypogastric nerves was a contraction of the detrusor muscle in the rat as is most often the case in other species (Giannuzzi 1863, Sherrington 1892, Langley and Anderson 1895, Mantegazza and Daimzada 1907, Vanoir (1965) did not obtain any effect of hypogastric nerve stimulation when recording the responses of the rat bladder as contractions in the axis from the urethra to the vertex instead of changes in the intravesical pressure. Some authors have found a relaxation of the bladder after an initial contraction in the dog and cat (Griffiths 1895, Kuntz and Saccomanno 1944). The response in the present study was not affected by the ganglionic blocking agent hexamethonium indicating that the fibres stimulated were postganglionic. Since the urinary bladder of the rat contains both excitatory  $\alpha$ -adrenoceptors and inhibitory  $\beta$ -receptors (Elmér 1973) an effect of adrenergic blocking agents on the response to hypogastric nerve stimulation could be expected. Neither the  $\alpha$ -receptor blocking agents dihydroergotamine or phenoxybenzamine nor the  $\beta$ -receptor blocking agent propranolol however had any significant effect.

on the detrusor contraction caused by hypogastric nerve stimulation. Furthermore the adrenergic neurone blocking drug guanethidine caused only a slight reduction of the response while atropine and the specific parasympatholytic agent Hoechst 9980 (Emmelin and Stromblad 1957) markedly reduced it. This is in agreement with the histochemical findings suggesting that the main part of the adrenergic nerves to the urinary bladder runs via the pelvic nerves. The slight reduction of the response to pelvic nerve stimulation caused by guanethidine also might support this concept although no effect of adrenoceptor blocking agents was found. The dose of guanethidine used was probably sufficiently low not to have any local anesthetic effect (Bein 1960). The existence of sympathetic fibres outside the hypogastric nerves was predicted by Giannuzzi (1863) on the basis of stimulation experiments in the dog.

The AChE positive nerves were clearly reduced at parasympathetic decentralization almost totally absent at total postganglionic denervation but apparently not changed in number in postganglionic sympathetic denervation. This probably means that most of the AChE positive nerves pass to the bladder via sacral nerve trunks and that a great part of them have their cellbodies located distant from the pelvic plexa. Furthermore it also suggests that the AChE positive nerves in the bladder wall are postganglionic fibres (cf. El Badawi and Schenk 1966).

The bladder response to both hypogastric and pelvic nerve stimulation was increased by eserine suggesting that cholinergic fibres were stimulated in both nerves. It is not likely that the potentiation of the response occurred at a ganglionic level since both the histochemical findings and the hexamethonium experiments indicated that the nerves stimulated in this case were postganglionic. When the pelvic nerve was stimulated proximal to the pelvic ganglion the response was not totally abolished by hexamethonium, indicating that some of the axons in the pelvic nerve originate from ganglion cells proximal to the pelvic ganglion in agreement with the histochemical findings.

Since the atropine resistance at hypogastric nerve stimulation was not greater than that of the pelvic nerve it is still possible that the nonadrenergic portion of the hypogastric nerve may be cholinergic. Even if this is the case the number of cholinergic nerve fibres in the bladder wall originating from the hypogastric nerves might be expected to be much smaller than that from the pelvic nerves and this may explain why no reduction of AChE positive nerves could be detected at sympathetic denervation.

Evidence of a non adrenergic mechanism involved in sympathetic nerve bladder transmission has been described in the cat (Sigg and Sigg 1964, De Sy 1972, Sundin and Dahlstrom 1973) and guinea pig (Mantegazza and Naimzada 1967). In these species the bladder wall contains intramural ganglia and atropine reduced the response only at such high doses that a ganglionic blocking effect can be expected (Feldberg and Vartiainen 1935). In the cat the hypogastric response is blocked by guanethidine and bretylium but not by the  $\alpha$  blocking agent phentolamine (De Sy 1972). An adrenergic stimulating action on cholinergic ganglion cells in the bladder wall was suggested, since adrenergic nerve terminals forming synaptic structures around non adrenergic ganglion cells have been demonstrated histochemically in the cat (Hamberger and Norberg 1965 a). In the rat bladder however where no evidence of intramural ganglia were found the non adrenergic part of the postganglionic symp



nerve bladder transmission might be explained by the presence of postganglionic cholinergic fibres in the hypogastric nerves. Hypothetically adrenergic nerve fibres may influence cholinergic nerve activity peripherally as described in the heart (Leaders 1963) the influence being inaccessible to adrenoceptor blocking agents.

The part of the response to hypogastric and pelvic nerve stimulation that could not be blocked by any of the sympathetic or parasympathetic antagonists used was totally abolished by the local anesthetic agent prilocain indicating that the bladder stimulation was nerve mediated and not due to any direct effect on the detrusor muscle.

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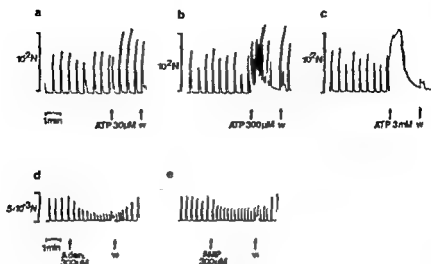


Fig. 1 a) b) and c) The effect of ATP on spontaneous mechanical activity in normal PSS. A low a medium and a high concentration is shown. d) and e) The effect of adenosine and AMP  $300 \mu\text{M}$  on spontaneous activity in normal PSS. Each substance is added at the first arrow and washed away at the second. Time and tension scales are shown by the bracketed bars.

expressed as the increase in mean tension in percent of maximal tension development induced by  $10 \mu\text{M}$  noradrenaline (NA). ATP was about 2.2 times more potent than ADP. At medium concentrations ADP sometimes lacked the initial contraction and only an increase in the frequency of contractions resembling the effect of AMP was seen.

The effect of ATP  $300$  or  $1000 \mu\text{M}$  was also tested for periods up to 15 min. It was found that after the initial excitation a short period of inhibition of mechanical activity followed. After 4–5 min however activity started anew with small contractions of high frequency resembling the effect of AMP (see below).

#### *The effect of AMP and adenosine on spontaneous activity in normal solution*

The effects of AMP and adenosine were qualitatively the same but opposite those of ATP and ADP. Both substances in concentrations from  $10 \mu\text{M}$  and upwards decreased the amplitude of the spontaneous contractions but increased their frequency (Fig. 1 d and e). The effects were graded according to dose and  $300 \mu\text{M}$  caused a 50% reduction in the contraction amplitude while the frequency of contractions was roughly doubled. Further increase in the drug concentration did not increase the response (see Fig. 2 b and Table I). The portal vein showed tachyphylaxis towards both AMP and adenosine when the interval between stimulations was less than 7–8 min or when the organ bath was only rinsed once following a medium or maximal concentration. In contrast to ATP and ADP there was also a slight decline in the response to AMP and adenosine during the course of the experiment. The inhibition caused by AMP or adenosine did not however change during the stimulation period even when the drug was present for periods up to 15 min. The effects of 5 different concentrations of AMP and adenosine on contraction amplitude are summarized in Fig. 2 b while Table I summarizes their effects on contraction frequency.

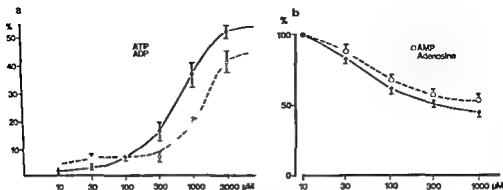


Fig 2 a) The dose response curves for ATP (filled circles) and ADP (open circles). Abscissa: Concentration in  $\mu\text{M}$ . Ordinate: Average tension during first min of response in percent of maximal tension response to  $10 \mu\text{M}$  NA. b) The effect of adenosine (filled circles) and AMP (open circles) on contraction amplitude in normal PSS. Abscissa: Concentration in  $\mu\text{M}$ . Ordinate: Contraction amplitude in percent of control. In both figures each point represents the mean of 8 muscles given with  $\pm$  S.E.

#### *The effects of ATP, ADP, AMP and adenosine on the K contracture*

When K high solution ( $128 \text{ mM K}^+$ ) is applied to the rat portal vein the muscle depolarizes and a smooth contracture develops. After a transient phasic peak contraction the contracture stabilizes at about 40% of the initial maximal tension (Axelsson *et al.* 1967). During the stable part of the contracture ATP, ADP, AMP and adenosine were applied at a concentration of  $300 \mu\text{M}$ . The contracture was allowed to stabilize after each stimulation. ATP and ADP increased the contracture while AMP and adenosine relaxed it. The increase in tension was for ATP  $19.1 \pm 4.4$  (4) and for ADP  $12.0 \pm 1.6$  (4) of baseline tension, while AMP relaxed the contracture by  $8.7 \pm 2.7$  (4) and adenosine by  $8.4 \pm 0.6$  (4).

#### *The effects of ATP, AMP and adenosine in solutions with varying $\text{Mg}^{++}$ and $\text{Ca}^{++}$ concentrations*

Reduction or removal of  $\text{Mg}^{++}$  from the normal PSS increases the amplitude and duration of the spontaneous contractions while the frequency is usually unchanged. When ATP  $300 \mu\text{M}$  was added in these conditions the drug effect was potentiated. Upon return to normal PSS the response to ATP decreased to control. On the other hand, when the  $\text{Mg}^{++}$  concentration was raised to  $4.8 \text{ mM}$ , i.e. 4 times normal, the spontaneous activity was drastically reduced and in a few cases completely inhibited. ATP in the same concentration as before had in this solution a very small effect.

A whole series of experiments were performed in which the  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  concentrations of the external medium were systematically varied. ATP  $300 \mu\text{M}$  was added for 3 min in each solution after the spontaneous activity had stabilized and the response to ATP after subtraction of the spontaneous activity in the corresponding solution compared to the maximal tension response to NA in the respective solution. The results are summarized in Fig. 3. From these results it is evident that the ATP effect is inversely related to the external  $\text{Mg}^{++}$  concentration. The ATP effect, however, was reduced in  $\text{Ca}^{++}$  high as well as in  $\text{Ca}^{++}$  low.

TABLE I The effect of AMP and adenosine on the frequency of contraction. All values expressed as the mean percentage increase in frequency caused by the drug  $\pm 2$  S.E. ( $n=5$ )

$\mu\text{M}$	10	30	100	300	1 000
AMP	—	$28 \pm 6$	$69 \pm 13$	$174 \pm 6$	$90 \pm 18$
Adenosine	$8 \pm 5$	$41 \pm 8$	$101 \pm 25$	$174 \pm 14$	$94 \pm 17$

PSS as compared to normal solution. Similar experiments were performed with adenosine and AMP but there were no changes in the effects of those substances neither with  $(\text{Mg}^{2+})_o$  nor with  $(\text{Ca}^{2+})_o$ .

The effects of ATP in varying  $\text{Mg}^{2+}$  concentrations were also studied on the h-contraction.  $\text{Mg}^{2+}$  free or  $\text{Mg}^{2+}$  high solutions *per se* did not shift the baseline of the contraction. When ATP 300  $\mu\text{M}$  was added the effect was in all solutions a transient contraction of constant magnitude. Thus in the depolarized tissue external  $\text{Mg}^{2+}$  had no influence on the ATP effect.

*Influence of ATP and adenosine on the response to exogenous noradrenaline and acetylcholine*  
*Acetylcholine (ACh)* ACh has an excitatory effect on the rat portal vein similar to the effect of NA causing a large summated contraction. To test if there was any interaction between ACh and ATP or adenosine these substances were added 2 min prior to 2  $\mu\text{M}$  ACh.

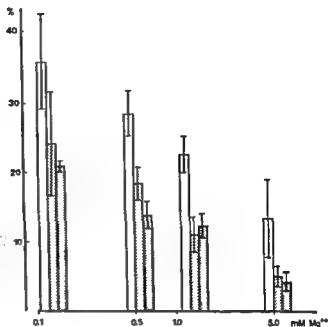
In the response compared to a control ACh application. It was found that the effect of ATP up to 1 mM was simply additive to that of ACh while adenosine partly inhibited the ACh contraction. The inhibition was 26–6% (5) for 300  $\mu\text{M}$  adenosine and 42–10% (4) for 1 mM adenosine.

*Noradrenaline (NA)* Similar experiments as with ACh were performed with 1  $\mu\text{M}$  NA. It was found that the effect of ATP was additive to that of NA. On the other hand adenosine or AMP did not as in the case of ACh cause partial inhibition. On the contrary the effect on NA was in some preparations potentiated while the normal response was an unchanged contraction in the adenosine pretreated muscle. The interaction between 1  $\mu\text{M}$  NA and 300  $\mu\text{M}$  adenosine was (also) tested in 4.0 mM  $\text{Ca}^{2+}$  and 0.5 mM  $\text{Ca}^{2+}$  PSS. It was found that in  $\text{Ca}^{2+}$  high solution the response to NA was significantly potentiated the ratio between adenosine treated and control stimulations being 1.15–0.04 (7). In  $\text{Ca}^{2+}$  low solution adenosine had no effect on the response to NA.

*Interaction of ATP, ADP, AMP and adenosine with  $\alpha$ -adrenergic and cholinergic blockers*

The effects of ATP, ADP, AMP and adenosine 300  $\mu\text{M}$  were studied in the presence and absence of phenoxybenzamine ( $\alpha$ -adrenergic blocker), propranolol ( $\beta$ -adrenergic blocker) or atropine (cholinergic, muscarinic blocker). The blockers were added at a concentration of 10  $\mu\text{M}$  which completely blocked all responses to added NA or ACh. The blockers had no effect either single or in combination on the response to ATP, ADP, AMP or adenosine.

Fig 3 The effect of Mg and Ca on the excitatory response to ATP 300  $\mu$ M in normal PSS (empty columns) Ca 0.5 mM (striped columns) and Ca 5 mM (hatched columns) The vertical bars represent  $\pm$  SE Abscissa  $Mg^{++}$  concentration in mM Ordinate A erage tension during first min of ATP response in percent of maximal contraction



### Effects of theophylline

Theophylline is known to be a potent phosphodiesterase inhibitor causing accumulation of 3'5' cyclic AMP in the cell. When theophylline 100  $\mu$ M was added and the response to ATP and adenosine 300  $\mu$ M after theophylline compared to control responses it was found that theophylline significantly potentiated the frequency increase caused by adenosine by  $14 \pm 4$  (6) and the decrease in amplitude by  $36 \pm 3\%$  (6). Theophylline *per se* slightly reduced spontaneous activity and did also decrease the response to ATP by about one third.

### The effects of related compounds on spontaneous activity and K contracture

To see if the shown effects were specific for the substances used GTP, GMP, guanosine, guanine, 3'5'-cyclic AMP and adenine were tested on the spontaneous activity and on the K-contracture in 4 preparations. The concentration for all substances was 300  $\mu$ M. GTP, guanine and 3'5'-cyclic AMP had virtually no effect neither in the polarized nor in the depolarized preparation. GMP, guanosine and adenine had a slight relaxing effect on the contracture and an AMP like effect on spontaneous activity. Guanosine 300  $\mu$ M was tested on spontaneous activity and compared to adenosine in the same concentration. Adenosine was found to be 5.0 times more potent in increasing contraction frequency and 2.7 times more potent in reducing contraction amplitude.

### Discussion

The results obtained in this study show that there is a difference between the effects of ATP and ADP on one hand and AMP and adenosine on the other. ATP and ADP always

an initial excitation while AMP and adenosine inhibited spontaneous activity. The direction of the response for the two groups of substances was independent of the ionic milieu and membrane polarization.

It is possible that the contraction induced by ATP in the rat portal vein is due to a direct effect on  $\text{Ca}^{2+}$  influx or  $\text{Ca}^{2+}$  release. Other possibilities should however first be evaluated. Thus ATP might interfere with the release of noradrenaline (or acetylcholine) from nerve endings but since none of the blockers tried affected the ATP response this is unlikely. The effects of ATP were also simply additive those of NA and ACh at the concentrations used above.

The question whether the effect of ATP could be due to energy liberation from the phosphate bonds was not posed in this study. However readmission of glucose after a period of glucose depletion inhibits spontaneous activity (Wahlström unpublished) and it is therefore not likely that energy liberation from ATP would cause an excitatory response.

ATP may bind  $\text{Mg}^{2+}$  in or at the membrane as proposed by Daniel and Irwin (1965). The results obtained in this study confirm this idea. In the rat portal vein the ATP induced contraction was obviously potentiated by decrease or removal of external  $\text{Mg}^{2+}$ . We also found however that the effect of ATP decreased in  $\text{Ca}^{2+}$  high solution irrespective of the  $\text{Mg}^{2+}$  concentration. This result might be explained by assuming that part of the ATP added is chelated by  $\text{Ca}^{2+}$  at this high  $\text{Ca}^{2+}$  concentration but it is more likely to be due to an increased membrane stability caused by increased  $\text{Ca}^{2+}$  binding at permeability-controlling sites in the high  $\text{Ca}^{2+}$  solution as proposed for guinea pig *tsenia coli* by Bülbürg and Tomita (1969).

The fact that ATP increases the  $\text{Ca}^{2+}$ -contracture the magnitude of the response being independent of the  $\text{Mg}^{2+}$  concentration could suggest an intracellular action of ATP. However as shown by Durbin and Jenkinson (1961) drugs—in their case Carbachol—can increase the  $\text{Ca}^{2+}$  uptake in smooth muscle and thus augment the tension by a membrane mechanism even in the depolarized condition.

AMP and adenosine had inhibitory effects on the rat portal vein except that adenosine potentiated the NA induced contraction on 5.0 mM  $\text{Ca}^{2+}$  solution. The latter results is similar to that obtained in the guinea pig vas deferens by Ito (1973) but the explanation for this phenomenon has yet to be found. Since AMP and adenosine hardly penetrate the cell membrane (Roll *et al.* 1956) the inhibitory effects in normal solution are probably exerted at the membrane and linked with the adenosine moiety. This might also explain why 3',5'-cyclic AMP had no effect in normal PSS because this compound is structurally different from AMP and adenosine.

The potentiation by theophylline of the AMP and adenosine effects indicates that an intracellular action is also possible. AMP and adenosine might through a membrane mechanism stimulate adenyl cyclase. This will increase  $\text{Ca}^{2+}$  binding to intracellular structures thereby decreasing the intracellular  $\text{Ca}^{2+}$  concentration (Andersson 1977). A similar mechanism decrease in  $[\text{Ca}^{2+}]_i$  was proposed to explain the effects of isoprenaline on the rat portal vein on the basis of the similarity between the effects of isoprenaline and low  $[\text{Ca}^{2+}]_o$  (Johansson *et al.* 1967). The records shown in fig. 1 d and e above also strongly resemble the effects of a moderate decrease in  $[\text{Ca}^{2+}]_o$ . The AMP and adenosine effects showed however

no correlation with external  $[Ca^{2+}]_0$  and the evidence for such a mechanism therefore is only tentative

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## Incorporation of Choline, Serine, Ethanolamine and Inositol into Phospholipids of Isolated Rat Mast Cells

By

KJELL STRANDBERG ANITA SYDBOM and BÖRJE ULLAS

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### Abstract

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The incorporation of labelled phospholipid precursors, [ $^3\text{H}$ ]-choline, L-[ $^3\text{-}^{14}\text{C}$ ] serine, L-[ $^3\text{-}^{14}\text{C}$ ] ethanolamine and L-[ $^3\text{H}$ ]-inositol into the phospholipids of isolated rat mast cells was studied. The label from the different precursors were found to be essentially associated with compounds with the following specificities for the respective phospholipids. Whereas the incorporation of L-[ $^3\text{Me-}^{14}\text{C}$ ] choline and L-[ $^3\text{S-}^{14}\text{C}$ ] serine showed evidence of saturation, the incorporation of L-[ $^3\text{C}$ ] ethanolamine was linear with time (h) and it was not saturated by increasing the concentration from 0.07 mM to 0.7 mM. The incorporation of L-[ $^3\text{H}$ ] inositol was stimulated by  $\text{Ca}^{2+}$  (1 mM) or  $\text{Mg}^{2+}$  (1 mM), while the incorporation of the other precursors was stimulated only in the presence of both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (1 mM). Antimycin A (1  $\mu\text{M}$ ), an inhibitor of the respiratory chain, significantly inhibited the incorporation of [ $^3\text{Me-}^{14}\text{C}$ ] choline, L-[ $^3\text{S-}^{14}\text{C}$ ] serine and L-[ $^3\text{H}$ ] inositol but not that of L-[ $^3\text{C}$ ] ethanolamine. The experimental system used might be a useful model for studies on the turnover of membrane phospholipids during histamine release.

Histamine release from rat mast cells can be induced by a variety of agents. Due to the morphological effects produced and/or the biochemical conditions required they have been classified as specific and non-specific releasers. Antigen and compound 48/80 are dependent on energy requiring reactions in the mast cell for their action. The precise nature of the release mechanism triggered by these agents remains to be ascertained. The participation of membrane bound enzymes, i.e. an esterase and a phospholipase A like enzyme and contractile elements in the process has however been implied (for references see Boeker and Henson 1973).

The appearance of biologically active lipid soluble principles on histamine release induced by antigen or compound 48/80 has been suggested to indicate the activation of a hydrolytic enzyme during the release (Ullas and Thörn 1969, Ånggård *et al.* 1963). Part of the active principles consists of prostaglandins (Byer and Vane 1969, Ånggård and Strandberg 1971), compounds known to be synthesized from fatty acids split off from membrane phospholipids by a hydrolytic enzyme (Lands and Samuelsson 1969). If a phospholipase

were activated in the mast cell during the histamine release changes in the phospholipid composition of the cells might be anticipated. In the present experiments the incorporation of labelled phospholipid precursors into rat mast cell phospholipids has been investigated in order to obtain a suitable experimental system for recording possible changes in the phospholipid pattern on histamine release.

## Materials and Methods

[Me- $^3$ C]-choline chloride (61 mCi/mmol) [  $^{14}$ C]-ethanolamine hydrochloride (30 mCi/mmol) L-[3- $^{14}$ C]-serine (48 mCi/mmol) and [2- $^3$ H]-myo-inositol (4.5 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. The radiochemical purity of the substances was tested by paper chromatography in different solvent systems.  $^{14}$ C-choline (99% purity) in butanol-acetic acid-water-ethanol 80:10:30:0 (v/v),  $^{14}$ C-ethanolamine (99%) and  $^{14}$ C-serine (99%) in butanol-acetic acid-water 20:30:50 (v/v),  $^3$ H-inositol (92%) in butanol-ethanol-water 104:66:30 (v/v).

Phosphatidylinositol, General Biochemicals, Chagrin Falls, Ohio, USA. Phosphatidylethanolamine and phosphatidyl-L-serine, Koch Light Lab. Ltd., England. L- $\alpha$ -Lecithin (synthetic), lysophosphatidylethanolamine and sphingomyelin, Sigma Chem. Comp. Inc., MO, USA.

Insta-Gel, Packard Instrument Comp. Inc., IL, USA. The scintillator solution toluene-PPO-POPOP consisted of 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-di-(2-(4-methyl-5-phenyloxazolyl))-benzene (POPOP) in toluene. PPO and POPOP were obtained from Koch Light Lab. Ltd., England.

Antimycin A was obtained from Nutritional Biochemical Corp., Cleveland, USA. It was dissolved in ethanol (96% v/v). The final concentration of ethanol in the incubation medium was 0.1% both in antimycin A and control samples.

Ficoll was purchased from AB Pharmacia, Uppsala, Sweden, and human serum albumin (free from preservatives) from AB Kab, Sweden. Solvents were Merck p.a. quality and were used without purification.

### Isolation of mast cells

Mast cells from the peritoneal and pleural cavities of male Sprague-Dawley rats (350–400 g) were isolated as described by Thon and Uvnäs (1967). After gradient centrifugation on Ficoll (350 g) the cells were washed twice at 20°C with a 9/1 (v/v) mixture of isotonic saline containing 1 mg of human serum albumin and 67 mM Sörensen's phosphate buffer, pH 6.3.

### Incubation procedure

An aliquot of the mast cell suspension containing about 2 million cells in 0.5 ml was incubated with 2  $\mu$ Ci of either  $^3$ C-choline,  $^3$ C-ethanolamine or  $^{14}$ C-serine or with 10  $\mu$ Ci of  $^3$ H-inositol in a water bath (10°C reversals/min) at 37°C for 30 min unless otherwise stated.

The standard incubation medium was a salt solution (NaCl 137 mM, KCl 4.7 mM,  $\text{CaCl}_2$  1.8 mM,  $\text{MgCl}_2$  1 mM) containing  $10^{-6}$  M Sörensen's phosphate buffer (67 mM), pH 7.0, human serum albumin (1 mg/ml) and glucose (1 mg/ml). The incubation was terminated by placing the tubes in ice water and adding 1.5 ml of the ice-cold medium.

### Extraction of phospholipids

After the incubation the cells were centrifuged (350  $\times$  g) at 4°C for 10 min. The supernatant was discarded and the cells were washed twice with 1 ml of the ice-cold medium. The radioactivity in the washings was counted to establish that non-incorporated material was eliminated. After washing the walls of the tubes were thoroughly dried by the use of strips of filter paper. One ml of isotonic NaCl was added and the cells were counted in a Barker chamber. 0.5 ml of distilled water was added to the cell suspension and the cells were sonicated at 4°C using a MSE ultrasonicator (8  $\mu$ m for 10 s). Aliquots (0.1–0.5 ml) of the suspension were taken for determination of radioactivity and protein (Lowry *et al.* 1951). The remaining suspension was extracted with 4.5 ml of chloroform-methanol (1/1 v/v) (Folch *et al.* 1957) containing 0.1 mg/ml of butylated hydroxytoluene (BHT) as an antioxidant (Wren and Szczerbanowska 1964) for 30 min at room temperature. The extract was washed with 9 ml of 0.6% NaCl and was left at 4°C overnight. The lower phase was collected and 33 ml of a theoretical mixture of lower phase was added to the upper

phase. After 5 h at 4 °C the lower phase was combined with the previous one. After evaporation the residue of the lower phase was dissolved in 2 ml of methanol and the residue of the upper phase in 2 ml of distilled water. The radioactivity was measured in both phases.

#### *Thin layer chromatography (t.l.c.)*

Glass plates (20 × 30 cm) were coated with a suspension of 40 g of Silica Gel H (E. Merck) in 90 ml of 1 mM Na<sub>2</sub>CO<sub>3</sub>, dried and then activated at 110 °C for 30 min. Extracted lipids (lower phase) were dissolved in chloroform and applied to a silicic acid column (2 g, 100 mesh, Mallinckrodt) with a diameter of 1 cm. 2 fractions were collected: chloroform 25 ml and methanol 25 ml. After evaporation of the methanol the residue was dissolved in a small volume (0.06–0.1 ml) of methanol and applied to a t.l.c.-plate 75/g amounts of reference phospholipids in 15 µl of methanol were also applied. The solvent system used was chloroform:methanol:acetic acid:water 50:30:8:4 (v/v) (Shlipsky *et al.* 1963) containing BHT 0.1 mg/ml. The chromatoplates were developed until the solvent front had reached 15 cm from the origin. The spots were detected with iodine vapor. The chromatoplates were divided into 1 cm zones and the silica gel was scraped off into liquid scintillation vials. The phospholipids were extracted from the silica gel with 0.5 ml of methanol before scintillation fluid was added. The recovery was about 80%.

#### *Determination of radioactivity*

Half a ml of the samples were transferred to vials and 10 ml of Insta Gel (for aqueous samples) or 10 ml of toluene PPO-POPOP (for methanol samples) was added. Packard Tri-Carb liquid scintillation spectrometer (model 3375) was used. The efficiency of the counting was estimated by using automatic external standardisation and curves for quenching. The incorporation of precursors into phospholipids was calculated by determining the radioactivity in the lower phase of the Folch extraction procedure. The incorporation was calculated as picomoles per million cells.

#### *Determination of lipid phosphorus*

Lipid phosphorus was determined according to the method of Bartlett (1959).

#### *Statistical method*

The Student's *t*-test was used for paired comparisons.

## Results

#### *Distribution of radioactivity*

The radioactivity was measured in both the upper (polar) and lower phases after the extraction of lipids from mast cells incubated with labelled precursors. The recovery of reference phosphatidylcholine and lysophosphatidylcholine in the lower phase averaged 96% as measured by determination of lipid phosphorus. Most of the label from incubations with <sup>14</sup>C-serine, <sup>3</sup>H-ethanolamine or <sup>3</sup>H-inositol and mast cells was recovered from the upper phase and only 23, 5 and 9 per cent respectively from the lower phase (Table 1). In contrast, as much as 56 per cent of the <sup>14</sup>C-choline added to mast cells was found in the lower phase.

#### *T.l.c. of the extracted phospholipids*

The <sup>14</sup>C-choline was found to be associated with a compound with the t.l.c. mobility of phosphatidylcholine (*R<sub>F</sub>* 0.64) and to a lesser extent (1%) with that of lysophosphatidylcholine (*R<sub>F</sub>* 0.17) with no detectable activity in the sphingomyelin area (*R<sub>F</sub>* 0.30). <sup>3</sup>H-inositol and <sup>14</sup>C-serine were likewise found to be incorporated essentially into the corresponding phospholipids, i.e. phosphatidylinositol (*R<sub>F</sub>* 0.83) and phosphatidylserine (*R<sub>F</sub>* 0.64).

TABLE I Distribution of radioactivity after lipid extraction of mast cells incubated for 30 min with labelled phospholipid precursors.

Precursor	c.p.m. per million cells <sup>a</sup>	
	Upper phase	Lower phase
<sup>14</sup> C choline	18 530 ± 610 (44 )	40 0 ± 2 610 (56%)
<sup>14</sup> C serine	21 000 ± 1 860 (77 )	6 090 ± 240 (23%)
<sup>14</sup> C ethanolamine	55 690 ± 3 640 (95 )	2 970 ± 130 (5 )
<sup>3</sup> H inositol	1 310 ± 50 (91%)	130 ± 5 (9 )

Means and standard errors (n=4) are given  
The distribution in per cent are given within brackets

0.90) Most of the <sup>14</sup>C-ethanolamine was associated with phosphatidylethanolamine ( $R_F=0.95$ ) but a minor amount (1%) of the label was recovered from an area with the same  $R_F$ -value as lysophosphatidylethanolamine ( $R_F=0.55$ )

#### Time course

The incorporation of the different precursors was followed for 2 h (Fig. 1). By that time the incorporation of <sup>14</sup>C-choline started to level off whereas the incorporation of <sup>14</sup>C ethanolamine and <sup>3</sup>H inositol continued in an essentially undiminished manner. In contrast the uptake of <sup>14</sup>C serine had reached its maximum level already after 1 h. Table II shows the incorporation of the different precursors after 30 min.

#### Effects of precursor concentration

Different amounts of unlabelled precursor were added to the incubation medium containing a constant amount of the labelled material (Fig. 2). The incorporation of <sup>14</sup>C-choline increased by 99 per cent when the concentration of choline was raised from 0.07 mM to 2.07 mM. The uptake of <sup>14</sup>C serine increased to a similar extent (51%) by increasing the concentration of the unlabelled precursor. In contrast there was a linear relationship between the concentration of ethanolamine added and the incorporation of <sup>14</sup>C-ethanolamine. The uptake increased by 575% when 2 mM of unlabelled ethanolamine was added. It was not possible to make a corresponding dilution of labelled material with myo-inositol because

TABLE II Incorporation of phospholipid precursors after 30 min incubation

Precursor	nmoles/10 <sup>6</sup> cells added	pmoles/10 <sup>6</sup> cells incorporated	% incorporation
<sup>14</sup> C-choline	3.0	288.7 ± 36.0	1.3
C-serine	18.6	7.6 ± 3.5	0.4
C-ethanolamine	41.4	69.1 ± 3.8	0
H-inositol	1.2	0.1 ± 0.01	0.0

Means and standard errors (n=4) are given

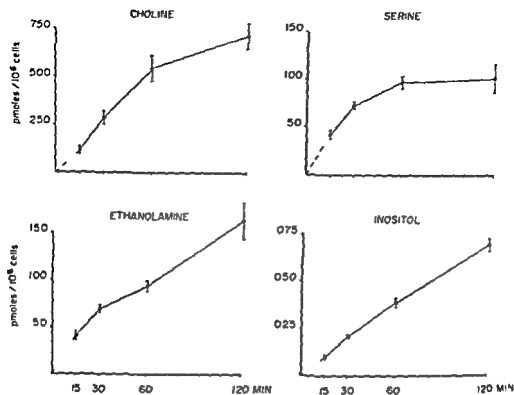


Fig. 1 Time course of the incorporation of labelled precursors  $^{14}\text{C}$ -choline ( $70\ \mu\text{M}$ )  $^3\text{H}$ -ethanolamine ( $10\ \mu\text{M}$ ) and  $^3\text{H}$ -inositol ( $5\ \mu\text{M}$ ). Means and standard errors ( $n=4$ ) are given.

the net incorporation of  $^3\text{H}$ -inositol (cpm) was just above the background value even though  $10\ \mu\text{Ci}$  was added.

#### Effect of $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$

In this series of experiments the incubation medium consisted of isotonic saline solution containing 10 mM Sørensen's phosphate buffer, albumin (1 mg/ml) and glucose (1 mg/ml). On addition of 1 mM  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  to the medium the incorporation of all precursors increased (Table III). Only in the experiments with  $^3\text{H}$ -inositol did 1 mM  $\text{Ca}^{2+}$  or 1 mM  $\text{Mg}^{2+}$  alone significantly promote the incorporation of the precursor.

#### Effect of antimycin A

Mast cells were preincubated for 5 min with antimycin A (1  $\mu\text{M}$ ) in a glucose-free medium. The labelled precursor was added and the incubation was continued for 30 min. Antimycin A was found to inhibit significantly the incorporation of  $^{14}\text{C}$ -choline,  $^3\text{H}$ -serine and  $^3\text{H}$ -inositol (Table IV). Addition of glucose (1 mM) to the medium *in* preincubated together with antimycin A partially counteracted the inhibitory effect of antimycin A (1  $\mu\text{M}$ ) as studied for the  $^{14}\text{C}$ -choline incorporation. Thus the inhibition was 47% and 90% respec-

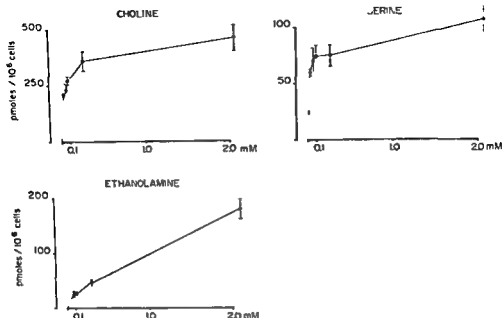


Fig. 2 Effect of precursor concentration on the incorporation. Different amounts of unlabelled precursor were added to 2  $\mu$  Ci of labelled precursor. Incubation time was 30 min.

tively with or without glucose present. The presence of glucose (1 mM) by itself was found to significantly stimulate the incorporation of <sup>1</sup>C-choline.

### Discussion

A great number of studies have been published on the incorporation of phospholipid precursors in different systems *e.g.* in rat liver subcellular particles *in vitro* (Kennedy and Weiss 1956, Dilis and Hubscher 1961, Bygrave and Bucher 1963, Bygrave and Kaiser 1968, Bjerve 1973) and *in vivo* (Spitzer *et al.* 1969, Bjerve and Bremer 1969, Treble *et al.* 1970).

TABLE III Effect of Ca<sup>2+</sup> (1 mM) and Mg<sup>2+</sup> (1 mM) on the incorporation of lipid precursors. Incubation time was 30 min.

Precursor	pmoles/10 <sup>6</sup> cells			
	Control	Ca	Mg <sup>2+</sup>	Ca and Mg <sup>2+</sup>
<sup>1</sup> C-choline	55.9 $\pm$ 4.0	70.9 $\pm$ 10.4	55.1 $\pm$ 5.0	97 $\pm$ 4.5
<sup>1</sup> C-serine	53.4 $\pm$ 5.4	70.9 $\pm$ 7.9	50.1 $\pm$ 3.5	78.6 $\pm$ 10.0
<sup>1</sup> C-ethanolamine	36.8 $\pm$ 3.6	44.0 $\pm$ 5.5	39.3 $\pm$ 4.4	59.8 $\pm$ 4.4
H inositol	0.06 $\pm$ 0.01	0.13 $\pm$ 0.01	0.15 $\pm$ 0.03	0.1 $\pm$ 0.0

Means and standard errors (n=4) are given.

0.01 < p < 0.05

0.001 < p < 0.01

p < 0.001 as compared to control values.

TABLE IV Effect of antimycin A (1  $\mu$ M) on the incorporation of phospholipid precursors

Precursor	pmoles $10^6$ cells <sup>a</sup>			
	Control	Antimycin A	Antimycin A + glucose (1 mM)	Glucose (1 mM)
<sup>14</sup> C-choline	115.9 $\pm$ 9.5 (10)	4.6 $\pm$ 1.4 (10)	61.3 $\pm$ 6.8 (6)	184.3 $\pm$ 20.0 (6)
<sup>14</sup> C-serine	55.5 $\pm$ 4.0 (4)	14.4 $\pm$ 1.3 (4)		
<sup>14</sup> C-ethanolamine	55.4 $\pm$ 6.9 (4)	36.3 $\pm$ 6.2 (4)		
<sup>3</sup> H-inositol	0.17 $\pm$ 0.04 (4)	0.04 $\pm$ 0.01 (4)		

<sup>a</sup> Means and standard errors are given. Figure without brackets represent number of determinations. 0.001 < p < 0.005,  $\square$  < 0.001 as compared to control values.

in chick brain microsomes (Porcellati *et al* 1971) in rat brain synaptosomes (Abdel Latif and Smith 1972) and in neoplastic mast cells (Pasternak and Bergeron 1970). Several of the authors reported a  $\text{Ca}^{2+}$  stimulated uptake of choline, serine and ethanolamine via base exchange mechanisms (Dils and Hubscher 1961, Porcellati *et al* 1971, Abdel Latif and Smith 1972, Bjerve 1973). In contrast the uptake of <sup>3</sup>H inositol in rat brain synaptosomes is inhibited by 1 mM  $\text{Ca}^{2+}$  and stimulated by  $\text{Mg}^{2+}$  and CTP suggesting that in this system the pathway for the incorporation of inositol is not by exchange (Abdel Latif *et al* 1973). The pathway for the incorporation of choline described by Kennedy and Weiss (1956) including phosphorylcholine and CDP-choline as intermediates for the incorporation of choline into phosphatidylcholine requires  $\text{Mg}^{2+}$  and CTP. The cholinephosphotransferase which transfers the phosphorylcholine moiety from CDP-choline to the diglyceride is inhibited by  $\text{Ca}^{2+}$  (about 1 mM) (Kennedy and Weiss 1956, Dils and Hubscher 1961, Hill and Lands 1971). In our experiments only the incorporation of inositol was significantly stimulated by 1 mM  $\text{Ca}^{2+}$  or 1 mM  $\text{Mg}^{2+}$ . Thus under the present conditions our data do not suggest a major role for base exchange with regard to the incorporation of choline, serine and ethanolamine in rat mast cells. For choline and serine this view is further corroborated by the findings that in rat brain synaptosomes the incorporation by base exchange does not seem to be dependent on energy yielding processes (Abdel Latif and Smith 1972). Thus in this preparation it was not blocked by metabolic inhibitors such as 2,4-dinitrophenol. In contrast we found that the incorporation of choline, serine and inositol was inhibited by antimycin A. This agent inhibits the electron transport between cytochrome b and  $\text{c}_1$  in the mitochondrial respiratory chain (for references see Wainio 1970). The observation that glucose partially counteracted the inhibitory effect of antimycin A on the incorporation of choline into the mast cells indicates that glycolysis to some extent can supply the energy required for this process.

The incorporation of ethanolamine differed from that of the other precursors studied. Thus it was linear during the experimental period and it was neither saturated by increasing the concentration from 0.07 mM to 2.07 mM nor blocked by antimycin A. This indicates that ethanolamine is transported into the cells via diffusion. Whether the energy dependency of the incorporation indicated for the other precursors reflects an active transport or energy requiring steps in the synthesis of the phospholipids can not be distinguished. It must be

recognized that our system involves intact cells in contrast to the cited studies on microsomes or mitochondria. It may be recalled that the uptake of *e.g.* choline into human blood platelets has been found to be energy requiring *i.e.* an active transport process has been indicated (Green *et al.* 1972).

The present study was principally undertaken to establish conditions for the incorporation of labelled precursors into rat mast cell phospholipids. This would set the conditions for an analysis whether changes in the phospholipid composition occur on histamine release from rat mast cells. From preliminary experiments it is clear that significant radioactivity appears in the incubates when mast cells labelled with  $^{14}\text{C}$ -choline,  $^{14}\text{C}$ -serine or  $^{14}\text{C}$ -ethanolamine are exposed to the histamine liberators *n*-decylamine and compound 48/80. Predominantly the base is recovered but also the corresponding phospholipids and lyso-compounds have been identified.

There are previous indications of an increased lipid turnover on histamine release from mast cells. Thus biologically active lipid soluble principles are formed on histamine release from isolated rat mast cells induced by compound 48/80 indicating hydrolysis of membrane lipids (Uvnäs and Thon 1959, Ånggård *et al.* 1963). Part of the biological activity recovered from the experiments with isolated mast cells (Ånggård *et al.* 1963) had characteristics similar to those of the prostaglandins. Hydrolysis of fatty acids esterified to phospholipids is regarded as the initial step in the biosynthesis of the prostaglandins (Lands and Samuelsson 1968). In fact it has been postulated that the activation of a phospholipase A like enzyme in the mast cell is an essential link in the histamine release process (Hogberg and Uvnäs 1957). Since isolated rat mast cells can incorporate phospholipid precursors we consider this system a suitable model to study the turnover of phospholipids during a release process. Such studies might have significance for the reactions of other secretory cells. Thus the histamine release and the related exocytosis triggered by agents such as antigen and compound 48/80 seems to operate via mechanisms similar to those activated when *e.g.* insulin and adrenal medullary amines are released (for references see Becker and Henson 1973).

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## Selective Localization of Histamine to Electron Dense Granules in Antigen-Challenged Sensitized Rat Mast Cells and to Similar Granules Isolated from Sonicated Mast Cells

An Electron Microscope Autoradiographic Study

By

PER ANDERSON and BÖRJE UVNAS

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### Abstract

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ANDERSON P and UVNAS B *Selective localization of histamine to electron dense granules in antigen challenged sensitized rat mast cells and to similar granules isolated from sonicated mast cells. An electron microscope autoradiographic study* Acta physiol scand 1975 94 63-73

The subcellular localization of histamine was studied in sensitized rat mast cells following antigen challenge and in granules obtained from sonicated cells using an electron microscope autoradiographic technique. The mast cells were furnished with labelled histamine by incubation in  $^3\text{H}$  histidine. The silver grain distribution (reflecting the localization of radioactive histamine) was highly non random. The highest silver grain densities occurred over homogeneous electron dense (normal) granules and moderately electron dense granules. Swollen less electron dense (changed) granules with a reticular appearance and devoid of a limiting membrane had the lowest density of all subcellular structures studied and were therefore probably almost free of histamine. There was a good correlation between the percentage of electron dense granules, the histamine content and the silver grain density in saline washed granule fractions isolated after sonication of mast cells for different times. These results support the hypothesis that histamine release occurs during the sequential exocytosis of storage granules and during the sonication of mast cells probably as a cation exchange between the amine which is ionically bound to the heparin protein complex of the granule matrix and cations from the extracellular fluid. The exchange will occur as soon as the perigranular membrane becomes permeable to water and cations.

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When mast cells are treated with a histamine releaser such as compound 48/80 or when sensitized mast cells are challenged with antigen *in vitro* characteristic ultrastructural changes occur in the cells during the histamine release process (Bloom and Haegermark 1965, Singleton and Clark 1965, Rohlich, Anderson and Uvnas 1971, Bloom and Chakravarty 1970, Anderson, Slorach and Uvnas 1973). Some of the granules which normally have a homogeneous electron dense (osmophilic) appearance acquire a swollen less electron dense reticular appearance. These changed granules which are found both outside and inside the cells are also devoid of a surrounding trilaminar membrane. By using extracellular tracer substances such as lanthanum or hemoglobin it has been established that all changed granules even those lying deep inside the cell in seemingly isolated pockets with no a

parent communication with the exterior, were in fact located in labyrinthic cavities formed during the release process and were in communication with the extracellular milieu (Röhlisch *et al* 1971, Anderson *et al* 1973). This finding was in agreement with the hypothesis of Uvnäs that the histamine release from all changed granules can be explained as a simple cation exchange between histamine bound to the heparin protein complex and cations in the extracellular fluid (Uvnäs and Thon 1967). Accordingly the histamine remaining in mast cells after exocytosis should be localized to the unchanged electron dense granules.

Isolation of granules from sonicated mast cells yields a mixed population of homogeneous electron dense granules and swollen less electron dense granules with a reticular appearance the former with a trilaminar membrane the latter devoid of a limiting membrane. On washing in saline the granules release part of their histamine to the suspension medium the higher the proportion of changed granules the greater the release (Anderson *et al* 1974).

The present electron microscope autoradiographic study demonstrates in a direct way the subcellular localization of histamine in both antigen-challenged sensitized mast cells and in saline washed granules obtained from sonicated mast cells. To be able to localize the histamine autoradiographically the granules were furnished with  $^3\text{H}$  histamine by incubating the mast cells *in vitro* with  $^3\text{H}$  histidine.

## Methods

### *Sensitivity procedure and preparation of cell suspension*

Male Sprague Dawley rats (180 g) were sensitized by a c. injection of 0.5 ml of egg albumin solution (100 mg/ml) and 0.5 ml of pertussis vaccine as adjuvant. 3 weeks later a mixed cell suspension was elicited from the peritoneum and pleura as described by Anderson *et al* (1971) using a salt solution ( $\text{NaCl}$  140 mM,  $\text{CaCl}_2$  2.0 mM) buffered with 10 mM Tris-Henrichsen buffer ( $\text{Na}_2\text{HPO}_4$  6.7 mM,  $\text{pH}$  7.4) and containing 0.1 mg of human serum albumin per ml.

### *Incubation with $^3\text{H}$ histidine*

The mixed cell suspension was centrifuged (100 g 5 min  $4^\circ\text{C}$ ) and the resulting sediment was then incubated for 1 h at  $37^\circ\text{C}$  in 1 ml of the above mentioned buffered salt solution containing 1 mCi  $^3\text{H}$  histidine (1 histidine 5.11 Spec. act. 44 Ci/mmole) giving a final histidine concentration of  $0.36 \cdot 10^{-6}$  M. The incubation was stopped by adding 8 ml of the salt solution ( $4^\circ\text{C}$ ) and the cells were washed twice before being resuspended in the salt solution to give a cell concentration of  $0.7 \cdot 10^6$  mast cells per ml.

### *Incubation with antigen*

Samples for electron microscope autoradiography and for histamine assay were run in parallel. 0.9 ml aliquots of the mixed cell suspension were preincubated at  $3^\circ\text{C}$  for 5 min before adding 5  $\mu\text{l}$  of egg albumin dissolved in 0.1 ml of the salt solution. The cells were incubated with 0.1 ml of the salt solution. Incubation was stopped after different times (10, 60, 90, 180 or 600 s) by adding either 9 ml of cold (4  $^\circ\text{C}$ ) glutaraldehyde = 0.1 M ionophosphate buffer ( $\text{Na}_2\text{HPO}_4$  11.0, 134 mM,  $\text{NaOH}$  107 mM,  $\text{pH}$  7.3) (electron microscope autoradiography) or 9 ml of ice cold buffer for histamine assay.

### *Electron microscope autoradiography of mast cells*

The samples were fixed for 10 min at  $4^\circ\text{C}$  and then for 1 h at  $25^\circ\text{C}$ . The cells were washed quickly in 1  $\text{O}_2\text{O}_4$  in 0.1 M Tris-Henrichsen buffer (45 min  $4^\circ\text{C}$ ), centrifuged and washed with 0.5 M Tris-Henrichsen buffer (pH 7.4) at  $4^\circ\text{C}$  suspended in a 1% gelatin solution and hydrated in an ethanol series, washed in propylene oxide and embedded in Araldite (for a detailed description of the procedure see Anderson *et al* 1973).

The sections were stained with a grey interference color (less than 16  $\text{\AA}$  thickness) according to Williams and Meeb (1962) were cut on a Reichert ultramicrotome OM U 34C (Reichert Optische Werke AG, Austria). The

sections were picked up on Formvar-carbon-coated 00 mesh copper grids (VECO Zeeplattenfabriek B V Eerbeek Holland) The grids with sections were attached with double coated tape (Scotch No 465 1/2 3 M Co St Paul, Minn) to microscope slides

Emulsion preparation and coating were performed by a modification of the technique described by Caro and Van Tubergen (196) and Caro (1969) Ilford L4 emulsion was melted at 45 C and diluted with an equal volume of distilled water The emulsion was cooled to 30 C Emulsion films were prepared by dipping a copper wire loop (inner diameter 4 cm) into the emulsion After the film had gelled in the air as shown by the change in the interference colour in reflected red light it was applied to the microscope slide carrying the grids The emulsion films applied to the grids were shown to consist of a monolayer of silver halide crystals as judged from the interference colour in white light and checked by electron microscopy after every coating session

Since no major difficulties were experienced in obtaining even monolayers of silver halide crystals from our Ilford L4 emulsion this technique was used instead of for example the more laborious "dipping" technique of Salpeter and Bachmann (1964) in which the monolayer is formed on a flat substrate provided by a microscope slide

The emulsion-coated sections were then exposed for 2-8 weeks at 4 C in sealed light tight plastic boxes containing Drierite They were then developed according to Caro and Van Tubergen (196) at 20 C in Kodak Microdol X diluted with 3 vol. of distilled water for 5 min washed in 1% acetic acid for 10 s fixed in Kodafix diluted with 3 vol of distilled water for 5 min washed in running tap water (5 min) and finally washed in distilled water (1 min) The grids were then separated from the microscope slides dried and stained with 2% aqueous uranyl acetate for 20 min at 60 C, followed by lead citrate for 15 min

Before the autoradiographs were analyzed it was confirmed that the grain pattern was not due to "background" or to positive chemography (production of a latent image by direct chemical action) by making autoradiographs of similarly prepared but unlabelled material Negative chemography (eradication of the latent image by direct chemical action) was checked by observing the behaviour of fogged emulsion layers overlying the sections

Since no chemical reaction was found between the sections and the emulsion in our material we did not cover the sections with a layer of carbon by vacuum evaporation before applying the emulsion as suggested by Jochler *et al* (1963) Bachmann and Salpeter (1964) and Salpeter and Bachmann (1964) According to Salpeter and Bachmann (1964 and 1972) this intermediate carbon layer is not essential for protecting the sensitivity of the more stable Ilford L4 emulsion as it is for other nuclear emulsions

The sections were observed in a Philips EM 300 electron microscope at 80 kV Semi thin sections for light microscopy were stained with a solution of toluidine blue and azur A containing 60% sucrose

#### *Preparation of mast cell suspensions in incubation with $^3\text{H}$ histidine and isolation of granules*

Mast cells were obtained from the peritoneal and pleural cavities of 10-12 male Sprague Dawley rats (350-400 g) using a buffered salt solution (NaCl 145 mM KCl 7 mM CaCl<sub>2</sub> 0.9 mM with 10 v/v Sørensen buffer (Na HPO<sub>4</sub> + KH<sub>2</sub>PO<sub>4</sub> 67 mM pH=7.0) and isolated by density gradient centrifugation in Ficoll as described by Anderson *et al* (1974) The cell suspension containing more than 90% mast cells was incubated with  $^3\text{H}$  histidine as described above for mixed cells After washing the isolated mast cells were resuspended in 25 ml of ice cold 0.34 M sucrose 5 ml aliquots cooled in ice were sonicated for 5 or 10 s with a MSE 100 W ultrasonic disintegrator (MSE Ltd, London England) set at 4  $\mu$ m amplitude using a 10 mm probe The sonicated suspensions were centrifuged (350 g 15 min 4 C) and the granule containing supernatants were then recentrifuged (3 000  $\times$  g 20 min 4 C) The granule pellets thus obtained were suspended in 0.1 M NaCl adjusted to pH 7.0 with Sørensen phosphate buffer and half of the suspensions was taken for electron microscope autoradiography and the other half for histamine heparin and protein assays The samples were centrifuged (3 000  $\times$  g 20 min 4 C) and the supernatants were discarded

#### *Electron microscope autoradiography of isolated mast cell granules*

The granule pellets were fixed with cold (4 C) 2.5% glutaraldehyde in Millonig phosphate buffer pH=7.3 for 40 min at room temperature and the granule suspensions were then centrifuged (3 000  $\times$  g 10 min 20 C) The granules were washed with Millonig phosphate buffer pH=7.3 and postfixed in 1% OsO<sub>4</sub> in the same buffer for 40 min at 4 C The granules were then centrifuged down (3 000  $\times$  g 10 min 4 C) before being dehydrated in an ethanol series, transferred through propylene oxide and embedded in Araldite

Thin sectioning, emulsion coating, exposure, development and staining were all carried out as described above for sensitized mast cells.

*Confirmation that histamine was the only significant radioactive compound in mast cells incubated in  $^3\text{H}$  histidine*

A pure mast cell suspension was obtained from the peritoneal and pleural cavities of 1-male Sprague Dawley rats by density gradient centrifugation in Ficoll as described above under "Preparation of mast cell suspension". A mixed cell suspension was collected as described above under "Preparation of cell suspension". The cell suspensions were incubated with  $^3\text{H}$  histidine (1 mCi Spec. act. 55 mCi/mmol, final histidine conc.,  $0.9 \times 10^{-3}$  M) in 2 ml of buffer for 1 h at 37°C. After washing, the cell sediments were suspended in 1 ml of buffer. 100  $\mu\text{l}$  of 3% HCl was added and the cells were heated at 80°C for 5 min. 10  $\mu\text{l}$  of this suspension was taken for ascending paper chromatography on Whatman no. 1 paper marked out in strips 4 cm wide. The following solvent system was used (all ratios by volume): n-butanol:pyridine:water 1:1:1. After drying, the chromatograms were cut into 1 cm lengths and, after cutting into smaller pieces, these were placed in counting vials together with 10 ml of Instagel scintillation liquid (Packard Instrument Co., Inc., La Grange, Ill. USA) and the radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co. Inc.). All results were corrected for quenching and were calculated as disintegrations per minute (DPM). Reference chromatograms for histidine and histamine were run using either  $^3\text{H}$  or  $^{14}\text{C}$  labelled compounds or using non-isotopic substances and then stained with ninhydrin. The radiochemical purity of the isotopic substances used was also checked by paper chromatography.

*Histamine, heparin and protein assays*

Histamine was assayed by the method of Shore, Burkhalter and Cohn (1959) as modified by Bergendorff and Urväs (1974). Heparin was measured by the amino-sugar method of Cessi and Piliego (1960) and is expressed in terms of a heparin standard. Protein was determined by the method of Lowry *et al.* (1951).

Histamine release is expressed as the percentage of the total histamine in the antigen treated sensitized cells. Histamine, heparin and protein were calculated as  $\mu\text{g}/\text{sample}$  in the isolated granule preparations and the results are presented as the histamine:heparin and histamine:protein ratios.

*Method of analysis of electron microscope photographs*

A large number of sensitized mast cells, both antigen treated and control cells, were photographed in the electron microscope. The electron plates obtained were printed on photographic paper at a final magnification of 70,000 times. Analysis of the silver grain distribution and the grain density was performed on prints from 10 mast cells treated with antigen for 10 min at 37°C. The exposure time for these cells was 6 weeks.

The method of analysis followed essentially that described by Williams (1969). Each silver grain on the micrographs was circumscribed by a circle the size of which was chosen to equal the resolution of our system, as defined by Barthman and Salpeter (1961), i.e. the circle had 50% probability of enclosing a site of the distribution. Using Lord's (1964) equation and assuming a section thickness of 100 Å, the electron has a radius of approximately 1.50 Å. According to Bachmann and Salpeter (1963):

Before starting the analysis of the tissue present in these 50% probability circles the different structural items of interest to be found in the mast cells were listed. Dense granules, moderately dense granules, less dense granules, cytoplasm and nucleus were considered as "single entities" (i.e. they could be localized completely inside them). "Junctional" items were combinations of the above mentioned structural entities with "junctions" or combinations of "single entities" with cytoplasm and nucleus. "Associated" items (i.e. groups of ultrastructural items smaller than the circle circumscribed together with their surroundings) were also included in the circles were also defined as "junctional" items.

Let us assume above classification the 310 silver grains observed from our material fell into 19 categories.

To find the relative number of each type of component (item) the effects of area of each item was calculated by the estimation of circles around on a clear transparent over each electron micrograph of the mast cell. 56 circles, 5  $\mu\text{m}$  in diameter corresponding to 50% probability circles at a magnification of 70,000 times.

To determine if the distribution was random or non-random a computer was made to select the circles and the grain frequency distribution was given by the frequency distribution of circles.

over the various ultrastructural features is an estimate of the frequencies of silver grains to be expected over those features if the grains were scattered randomly)

The specific activities (that is the number of silver grains per unit area feature (silver grains/ $\mu^2$  circles) were calculated for the different ultrastructural stems in the mast cells.

Electron microscope autoradiographs of granules isolated from mast cells incubated in  $^3\text{H}$  histidine and then sonicated were analyzed using the same procedure except that the specific activities were expressed in silver grains per granule.

## Materials

Araldite (Durocan ACM) FLUKA AG Buchs SG Switzerland Egg albumin (Disco Laboratories Inc Detroit Michigan USA Ficoß AB Pharmacia Uppsala, Sweden Glutaraldehyde (25% especially purified for electron microscopy) TAAB Laboratories Reading, England Heparin sodium (pig mucous) containing 9.2 per cent sulphur AB Vitrum Stockholm Sweden Human serum albumin (free from preservatives) AB Kabi Stockholm Sweden Ilford L4 emulsion Ilford Ltd Essex, England Pertussis vaccine ( $0 \times 10^6$  bact per ml) SBL Stockholm Sweden L-( $^3\text{H}$  histidine), Spec act 55 Ci/mmol (Batch 29) and 58 Ci/mmol (Batch 27) and histamine (ring  $^3\text{C}$ ) Spec act 59 mCi/mmol Amersham England All other substances were obtained from the usual commercial sources

## Results

### *Fate of the radioactivity during preparation of the cell suspension for electron microscopy*

In order to determine possible extraction of the isotope during preparation for electron microscopy the radioactivity in the solutions used at the different steps in the preparation procedure was measured in three experiments with mixed cell suspensions. After glutaraldehyde fixation and washing, not more than 0.2–1.6% of the total radioactivity was lost during osmium tetroxide fixation washing ethanol dehydration and propylene oxide soaking. Thus almost all the radioactivity taken up by the cells during the  $^3\text{H}$  histidine incubation was still present in the cells when embedded in Araldite.

### *Fate of $^3\text{H}$ histidine in pure mast cell suspension*

When a pure mast cell suspension was incubated with  $^3\text{H}$  histidine (1 mCi Spec act 55 Ci/mmol histidine conc  $0.9 \times 10^{-6}$  M  $4.5 \times 10^6$  mast cells/ml) for 1 h at 37°C using the same method as used for sensitized mixed cell suspensions prior to em autoradiography more than 90% (91–96%) of the radioactivity was found to be  $^3\text{H}$  histamine as shown by paper chromatography.

### *Sensitized mast cells treated with antigen*

**Histamine release** When  $^3\text{H}$  histidine incubated mast cells in a mixed cell suspension from peritoneum and pleura from sensitized rats were challenged with antigen (5  $\mu\text{g}$  egg albumin/ml) for different times at 25°C the histamine release started after a latent period of 30 s increased to 14% after 3 min and reached a maximal value of 16% after 10 min in the present em autoradiography experiment.

**Morphology** The changes observed in the morphology of sensitized mast cells during antigen induced histamine release have been described in detail earlier both at the light and at the electron microscopic level (Anderson *et al* 1973) and are therefore omitted. It is sufficient to point out that when viewed in the light microscope cells treated for longer times contain more and more granules which stain pink with a toluidine blue azur A.

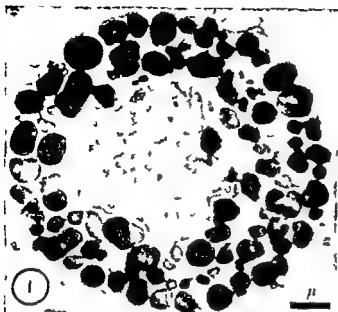


Fig 1 Sensitized mast cell treated with antigen for 10 min at 5°C. The cytoplasm is filled with homogeneous electron dense granules. No signs of any ultrastructural changes can be seen in this cell. Most silver grains are found over the dense granules. Magnification  $\times 9\,500$

tion instead of dark blue the colour associated with granules in untreated cells. In the electron microscope these changed granules had a swollen less electron dense reticular appearance in contrast to the homogeneous electron dense appearance of normal granules in unchallenged cells.

**Grain distribution** Silver grains were found over both the mast cells and the leucocytes in our mixed cell suspension. In the mast cells most grains were found over the electron dense granules but some were also found outside the granules some over the nucleus (Fig. 1 and Fig. 2).

To establish if the grain distribution was random or non random a comparison between the grain distribution and the circle distribution was made using a  $\chi^2$  test. The grain distribution was found to be highly non random ( $\chi^2 = 118.07$   $P = 0.001$ ) which is also evident from Table I.

The silver grain densities of the different subcellular structures (items) in the mast cells are seen in Table I. Dense (normal) granules had the highest grain density (% silver grains/ $\mu^2$  circles) (3.87) followed by moderately dense granules (2.85) while less dense (changed) granules showed the lowest density (0.13). Junctional items such as dense granules cytoplasm and moderately dense granules cytoplasm had grain densities between those of the corresponding single items.

The ratios between the silver grain densities of different subcellular structures are shown in Table II. The ratio between dense granules and less dense granules was found to be 29.77 which means that dense granules contain about 30 times more radioactivity than less dense granules. Moderately dense granules contained about 20 times more radioactivity than less dense granules. Dense granules contained 3.5 times more label than cytoplasm or nucleus while less dense granules had only 1/9 or 1/6 of the label of the cytoplasm or the nucleus.



Fig 2 Sensitized mast cell treated with antigen for 10 min at 25°C. Many "changed" swollen less electron dense granules lying in the cell can be observed. Some granules (X) have an electron density between that of the normal electron dense granule and that of the changed less electron dense granule. Liver grains are mainly found over dense granules. Less electron dense granules are almost devoid of grains. Magnification  $\times 9,300$ .

#### Granules isolated from sonicated mast cells

Pure granule fractions were obtained from  $^3\text{H}$  histidine incubated mast cells by sonication at  $4\ \mu\text{m}$  amplitude for either 5 or 120 s.

**Morphology** Two types of granule were found. One type was homogeneous, electron dense and surrounded by a trilaminar membrane. The other type was less electron dense and had a swollen, reticular-like structure and was devoid of a limiting unit membrane (Fig 3).

TABLE I Analysis of the grain distribution on em autoradiographs of sensitized rat mast cells incubated with  $^3\text{H}$  histidine prior to inducing histamine release by antigen challenge for 10 min at 25°C.

Item	silver grains	circles	# silver grains/ circles
Dense granules	35.65	9.22	3.87
Moderately dense granules	2.74	0.96	2.85
Less dense granules	3.87	29.70	0.13
Nucleus	10.37	12.79	0.81
Cytoplasm	7.4	6.53	1.14
Dense granules/Cytoplasm	9.84	19.40	1.54
Moderate granules/Cytoplasm	1.94	0.84	31
Less dense granules/Cytoplasm	10	5.53	0.38
Dense granules/Nucleus/Cytoplasm	1.94	1.40	1.39
Other items	4.18	13.63	0.31
(310 silver grains) (495 circles)			

Comparison of the silver grain distribution with the circle distribution:  $\chi^2 = 118.07$ ,  $P < 0.001$ , i.e. the grain distribution is highly non-random.





Fig 3 High power em autoradiograph of granules obtained by sonication of  $^3\text{H}$  histidine incubated mast cells at 4  $\mu\text{m}$  amplitude for 5 s. Note that the electron dense granules are surrounded by a trilaminar membrane that can be seen at favourable planes of sectioning (arrows). Trilaminar membrane can be seen around (changed) less electron dense granules. Magnification 30 000.

At the shorter sonication time 51% of the granules were found to be dense granules compared to 17% at the longer sonication time.

**Histamine content** The histamine/heparin and histamine/protein ratios were found to be higher at the shorter sonication time (0.11 and 0.15 respectively means of 4 samples taken in parallel with the em autoradiography) than at the longer time when both ratios had decreased to 0.04.

**Grain distribution** The silver grain distribution was analyzed in 7 autoradiographs taken from the granule fraction obtained after 5 s sonication and from as many prints from the experiment with 120 s sonication. 213 silver grains were found in totally 1544 granules. At 5 s sonication time the ratio for grains per granule (grain density) was found to be 0.20. At 120 s sonication time the ratio had decreased to 0.07 (Fig. 4). The silver grain density for dense granules was 0.33 and for less dense granules 0.05 (Table III).

TABLE II Analysis of the ratio between the grain density of different items on em autoradiographs of sensitized rat mast cells incubated with  $^3\text{H}$  histidine prior to inducing histamine release by antigen challenge for 10 min at 37°C

	Density ratio	Sample estimates of the standard deviation	t value	df	p
Dense granules	2.77	5.93	0.10	17	0.91
Less dense granules					
Dense granules	1.6	5.93	1.85	11	0.08
Moderately dense granules					
Dense granules	4.8	5.93	0.51	18	0.61
Nucleus					
Dense granules	1.2	5.23	1.85	17	0.05
Cytoplasm					
Moderately dense granules	1.2	1.85	0.10	6	0.9
Less dense granules					
Moderately dense granules	1.18	1.85	0.55	11	0.61
Nucleus					
Moderately dense granules	50	1.85	1.85	10	0.03
Cytoplasm					
Less dense granules	11	0.10	0.55	8	0.6
Nucleus					
Less dense granules	1	0.10	1.85	11	0.10
Cytoplasm					

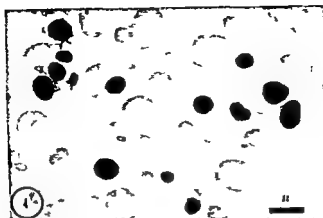


Fig. 4. Em autoradiograph of a pure granule fraction obtained from  $^3\text{H}$  histidine incubated mast cells sonicated at  $4\ \mu\text{m}$  for 1.0 s. The silver grains are mostly associated with the electron dense granules. Magnification  $\times 8,800$ .

### Discussion

A mixed cell suspension was used in the present electron microscope autoradiographic study instead of isolating the mast cells on a Ficoll gradient. The time of handling the cells prior to fixation could therefore be shortened.

When a pure mast cell suspension was incubated with  $^3\text{H}$  histidine more than 90% of the radioactivity was found to be present as histamine. Similar findings have been reported by Cabot and Haegermark (1968). At least 90% of the silver grains overlying the mast cells in our cell suspensions must therefore be due to the presence of labelled histamine.

The histamine release from sensitized mast cells increased progressively with increasing incubation time after antigen challenge. Simultaneously some of the normal dark blue staining granules in the mast cells were stained pink (metachromatic) with toluidine blue azur A in the light microscope. In the electron microscope these "changed" granules had a swollen less electron dense reticular appearance instead of the homogeneous, electron dense feature of normal granules. At the shorter incubation times there were relatively few "changed" granules and these were mostly located near the cell periphery. The number of changed granules increased with longer incubation times and they could then be found seemingly deep inside the cells.

TABLE III: Means and standard deviations of the silver grain density (silver grains/granule) in dense and less dense granules isolated from mast cells incubated in  $^3\text{H}$  histidine and sonicated at  $4\ \mu\text{m}$  amplitude setting for 5 and 1.0 s and  $t$ -value for the differences obtained.

	Mean	Sample estimate of the standard deviation	$t$ value	df	$P$
Dense granules	0.33	0.10	9.1	5	<0.001
Less dense granules	0.05	0.04			

13 silver grains were analyzed in 1,544 granules on 16 autoradiographs from both incubation times.

The silver grain distribution was highly non random in antigen-challenged sensitized mast cells. The highest grain density reflecting the highest amount of labelled histamine was found in the electron dense (normal) granules. The lowest density was detected in less dense (changed) granules. Moderately dense granules which are granules with an electron density between that of normal and "changed" granules were also sometimes found. Similar granules described as slightly swollen granules have been observed earlier when mast cells were incubated with compound 48/80 (Röhlich *et al.* 1971) and when sensitized mast cells were treated with antigen (Anderson *et al.* 1973). These granules are often situated close to the extracellular space and might therefore represent granules releasing histamine during an influx of cations and water by the ion-exchange mechanism suggested by Uvnäs and collaborators.

The analysis of the silver grain distribution thus clearly showed that the greater part of the radioactive histamine was localized in dense or moderately dense granules or in junctional structures including these granules. The smallest amount of histamine was found in less dense (changed) granules. The fact that some grains were found outside dense and moderately dense granules in other subcellular structures can be explained as being partly to the scattered tail of the distribution curve for the silver grains and possibly partly due to the presence of a small amount of unchanged radioactive histidine. The presence of labelled histidine has in fact been described in other cell types after glutaraldehyde fixation for em autoradiography (Salpeter and Bachmann 1972).

In order to compare the distribution of histamine and the ultrastructural changes in mast cell granules without interfering structures granules were isolated from  $^3\text{H}$  histidine incubated mast cells.

Sonication for 5 s gave a granule fraction containing 51% electron dense granules. The granule fraction had a histamine content of 0.11 when expressed as the histamine/heparin ratio (see Methods for definitions of ratios) or 0.15 when expressed as the histamine/protein ratio and a total grain density of 0.10.

Sonication for 120 s gave a granule fraction containing 17% electron dense granules. This fraction had a granule content of histamine of 0.04 (histamine/heparin and histamine/protein ratios) and a total grain density of 0.07.

Thus it is evident that there exists a good correlation between the percentage of electron dense granules, the granule content of histamine and the total grain density (reflecting the amount of histamine per granule) when the granule fractions obtained after the two different sonication times are analyzed.

Calculation of the grain densities for isolated dense granules (0.33) and less dense granules (0.05) showed that dense granules with an intact perigranular membrane contained at least 7 times more radioactive histamine than the less dense granules.

In the present em autoradiographic investigation it was thus possible to show a selective localization of histamine to electron dense granules in antigen-challenged sensitized mast cells and to similar granules isolated from sonicated mast cells. "Changed" granules on the other hand had the lowest grain density of all subcellular structures in the mast cell and could be regarded as almost free of histamine.

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The silver grain distribution was highly non random in antigen-challenged sensitized mast cells. The highest grain density reflecting the highest amount of labelled histamine was found in the electron dense (normal) granules. The lowest density was detected in less dense (changed) granules. Moderately dense granules which are granules with an electron density between that of normal and changed granules were also sometimes found. Similar granules described as slightly swollen granules have been observed earlier when mast cells were incubated with compound 48/80 (Rohlich *et al.* 1971) and when sensitized mast cells were treated with antigen (Anderson *et al.* 1973). These granules are often situated close to the extracellular space and might therefore represent granules releasing histamine during an influx of cations and water by the ion-exchange mechanism suggested by Uynäs and collaborators.

The analysis of the silver grain distribution thus clearly showed that the greater part of the radioactive histamine was localized in dense or moderately dense granules or in junctional structures including these granules. The smallest amount of histamine was found in less dense (changed) granules. The fact that some grains were found outside dense and moderately dense granules in other subcellular structures can be explained as being partly to the scattered tail of the distribution curve for the silver grains and possibly partly due to the presence of a small amount of unchanged radioactive histidine. The presence of labelled histidine has in fact been described in other cell types after glutaraldehyde fixation for *em* autoradiography (Salpeter and Bachmann 1972).

In order to compare the distribution of histamine and the ultrastructural changes in mast cell granules without interfering structures granules were isolated from  $^3\text{H}$  histidine incubated mast cells.

Sonication for 4 s gave a granule fraction containing 51% electron dense granules. The granule fraction had a histamine content of 0.11 when expressed as the histamine/heparin ratio (see Methods for definitions of ratios) or 0.15 when expressed as the histamine/protein ratio and a total grain density of 0.10.

Sonication for 120 s gave a granule fraction containing 17% electron dense granules. This fraction had a granule content of histamine of 0.04 (histamine/heparin and histamine/protein ratios) and a total grain density of 0.07.

Thus it is evident that there exists a good correlation between the percentage of electron dense granules, the granule content of histamine and the total grain density (reflecting the amount of histamine per granule) when the granule fractions obtained after the two different sonication times are analyzed.

Calculation of the grain densities for isolated dense granules (0.33) and less dense granules (0.05) showed that dense granules with an intact perigranular membrane contained at least 7 times more radioactive histamine than the less dense granules.

In the present *em* autoradiographic investigation it was thus possible to show a selective localization of histamine to electron dense granules in antigen-challenged sensitized mast cells and to similar granules isolated from sonicated mast cells. Changed granules on the other hand, had the lowest grain density of all subcellular structures in the mast cell and could be regarded as almost free of histamine.

Robinson *et al* 1967) Since these lines of work turned out to be negative a more general screening was initiated

Part of this work was presented at a Neurosciences Research Program Work Session Boston May 1974 (Terenius 1975 a) and at the 28th Scandinavian Pharmacologic Society Meeting Odense August 1974 (Terenius and Wahlstrom 1974)

## Materials and Methods

**Substances and reagents** Dihydromorphine (DHM) labelled with tritium by catalytic hydrogenation of morphine at a specific activity of 55-60 Ci/mmol was obtained from New England Nuclear Corp Boston Mass Unlabelled DHM was prepared by the same procedure by the author The peptides for column calibration were purchased from Sigma Co St Louis Mo All other chemicals were of reagent grade

Receptor preparations from synaptic plasma membranes of rat brains were obtained as described earlier (Terenius 1974) The receptor preparation from the guinea pig ileum also followed a previously described procedure (Terenius 1973 c) These preparations were obtained on a large scale basis and aliquots were kept frozen at  $-70^{\circ}\text{C}$  for up to 6 months without reduction in receptor content

The HEPES buffer had the following composition NaCl 124 mM KCl 5 mM  $\text{KH}_2\text{PO}_4$  1 mM CaCl 0.75 mM N 2 hydroxyethyl piperazine N 2-ethanesulfonic acid HEPES 26 mM It was adjusted to pH 7.4 at  $25^{\circ}\text{C}$

The liquid scintillation cocktail was toluene 3 1 2 butoxyethanol 60 ml PPO 15 g and dimethyl POPOP 0.9 g

**Screening of brain extracts for receptor affinity** A method was required that could be used for a large number of crude extracts and more or less purified fractions The receptor preparation had to be as free as possible from degradative enzymes A technique already described (Terenius 1974) meets these criteria (1) it utilizes the synaptic plasma membrane (SPM) fraction of rat brain from which enzymes in cytoplasm and mitochondria are removed during the processing (2) it is not very sensitive to the eventual presence of inorganic salts since the assay is run in a physiologic buffer (3) it is rapid and a large number of samples can be run simultaneously

All extracts and fractions were first lyophilized and then made up in HEPES buffer before the receptor assay Of the extract or fraction 0.1 ml was added to 0.3 ml of HEPES buffer with SPM (or ileum preparation) corresponding to about 0.4 mg of protein and labelled dihydromorphine to a final concentration of  $0.8 \times 10^{-10}$  M The mixture was incubated in a reciprocating incubator at  $25^{\circ}\text{C}$  for 40 min The incubation was terminated by centrifugation in the cold for 10 min in a Microfuge (Beckman) The SPM pellet was obtained by cutting the tip of the tubes It was digested by 1 ml of Soluene (Packard) toluene (1:3 v/v) Liquid scintillation counting of the radioactivity of the pellets and of the medium aliquots followed Each experimental run included the following samples

Sample	DHM	Extract	Nonlabelled DHM (final concentration $10^{-10}$ M)
Control	+	—	—
Experimental	+	+	—
Carrier	+	—	+

Control or carrier samples contained 0.1 ml of HEPES buffer instead of extract At least 7 to 8 samples of each kind or each concentration were run in an experiment

The purpose of including the carrier samples was to correct for nonspecific (nonspecific) binding Values for carrier samples were subtracted from control and experimental values The effect of a fraction on receptor binding is then obtained in percent of the corrected control binding ( $\sim 100\%$ ) By the use of a log concentration inhibition curve which was similar for many preparations (Fig. 1) the inhibition value was transformed to relative concentration of receptor blocker

**Preparation of brain extracts (cf Fig. 1)** Female Sprague Dawley rats weighing about 125 g were exsanguinated decapitated and the whole brain except the cerebellum was taken into ice-cold Potter Elvehjem homogenizers The brains were thoroughly homogenized with two volumes of ice-cold 0.1 M buffer of pH 4.0 The homogenate was deproteinized by heating at  $70^{\circ}\text{C}$  for 20 min centrifuged

filtered through a PM10 (Amicon) filter (nominal cut off 10 000 dalton). After the heating all further purification steps were carried out at 0–+4 °C.

**Chromatographic parameters.** 20 ml of the ultrafiltrate adjusted to pH 2.9 with HCl was run on a carboxymethyl cellulose ion exchanger (CM5<sup>+</sup>, Whatman 13×2 cm) pre-equilibrated with 0.1 M acetic acid. Stepwise elution with about 20 ml of 0.2 M acetic acid, 150 ml of 1 M acetic acid and 200 ml of 5 M acetic acid followed. The eluent was monitored in an Uvicord (LKB Beckman) at 254 nm and about 50 ml of the break through peak of 1 M acetic acid (Fraction A) and 70 ml of the corresponding peak of 5 M acetic acid (Fraction B) were lyophilized. Fraction A was redissolved in 1 ml of water and run on a PGM 7000 (Merck, 100×0.9 cm) column in water. The effluent was continuously monitored in an Altex Model 150 Biochemical UV analyzer set at 254 nm (Fig. 4).

**Analyses.** Concentrations of Na<sup>+</sup> and K<sup>+</sup> were measured with an Eppendorf flame photometer against factory standards. Assays for primary amines with fluorescamine (Fluoram, Roche) were carried out at pH 8.5 (Udenfriend *et al.* 1977). A standard curve with leucine was included on each experimental occasion.

## Results

**Initial screening of brain extracts.** Since the extracts were to be tested in a biologic system extreme pH or ionic strength were not allowed. An ideal way to circumvent inorganic salts would be extraction into organic solvents which could be evaporated later. As mentioned in the introduction a number of attempts were made to isolate receptor binding blockers in chloroform by extraction of brain homogenates at different pH. No significant receptor blocking activity was found in concentrated extracts from 5–10 rat brains. When we followed the extraction scheme of Green and co-workers no binding activity was found in the petroleum ether extract where they found the active substances by bioassay (Green *et al.* 1963; Robinson *et al.* 1967). On the other hand the water phases were slightly active in our system (and inactive in theirs). Subsequent work was directed to water soluble fractions.

Water extracts were obtained as described in Methods. After highspeed centrifugation ultrafiltration with different Amicon filters was applied. Even if we used filters with a nominal cut off of 1 000 dalton there was no evidence of accumulation of receptor blocking factors in the retentate (concentration factor 10–20 fold). In all subsequent work a filter with a nominal cut off of 10 000 dalton was used to speed up the filtration. The next step in the isolation procedure was ion exchange chromatography.

**Isolation of a receptor blocking endogenous factor.** An initial desalting step was accom-

TABLE 1 Chemical characterization of the different fractions of the purification procedure. Mean values from several experiments are shown. The values are given per one rat brain (1.4 g wet weight).

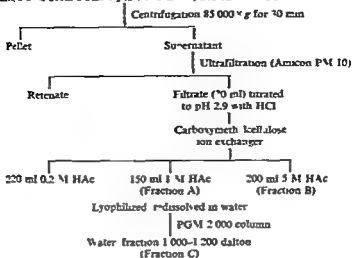
Fraction	Absorbance units (260 nm)	Primary amine groups (meq)	Na <sup>+</sup> (meq)	K <sup>+</sup> (meq)	Receptor activity (units) <sup>a</sup>
Extract (after ultrafiltration)	50	0.34	0.2	0.1	
Fraction A	0.57	$3 \times 10^{-5}$	ND <sup>c</sup>	ND	0.27
Fraction C	0.021	$7.5 \times 10^{-5}$	ND	ND	0.18

<sup>a</sup> Fluorescamine reaction, leucine equivalents.

<sup>b</sup> For reasons given in the text, receptor activity of the crude extract is not measurable. Unfused

<sup>c</sup> ND = not detectable.

Homogenate (brain) 0.1 M acetate buffer pH 4.0 (1.2 w/v) heated to 70°C for 20 min





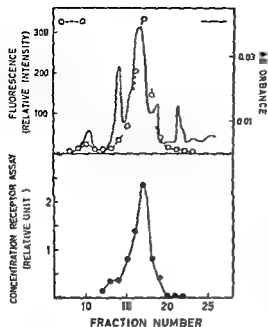


Fig. 4 The upper part gives the elution profile from the PGM 4000 column in terms of fluorescence after reaction with fluorescamine (Fluoram Roche which reacts with primary amines) and UV absorption at 254 nm. The lower part gives the receptor blockade in relation to elution volume. The units for receptor blockade are those of Fig. 2.

amino acids of known molecular weight (Fig. 3). Fraction B was more complex than Fraction A and was not characterized further. The PGM column effluent from the runs with Fraction A were continuously monitored for UV absorption and also analyzed for primary amines by reaction with fluorescamine. The results (Fig. 4) indicated a coincidence between fluorescamine positive reaction and receptor blocking activity. Some of the UV absorbing material was fluorescamine negative and showed no receptor blocking activity.

The large UV peak which almost coincided with the fluorescamine positive peak had an absorption maximum at 273 nm. It also gave a fluorescence spectrum (excitation maximum 280 nm, emission maximum 320 nm). The fluorescence intensity was highest at neutral pH but very much less at pH 1.5 and somewhat less at pH 11, a typical characteristic for the

Fig. 5 Double reciprocal analysis of inhibition of dihydromorphine binding to synaptic plasma membrane receptor by the endogenous factor. The lower curve is the control, the upper curves with two concentrations of factor.

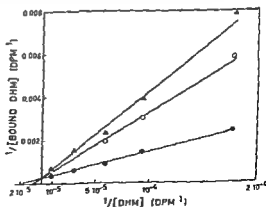
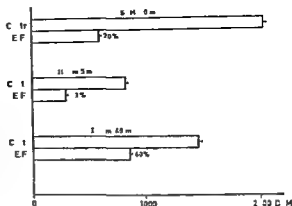


Fig 6 Effect of the endogenous factor (E.F.) on dihydromorphine binding to the opiate receptor of synaptic plasma membranes (SPM) and the guinea pig ileum. With ileum incubation time was either 5 or 40 min. The inhibition is given in percent of control (Contr)



aromatic amino acids tryptophan and tyrosine (Shore and Pardee 1956). The receptor blocking activity was not destroyed by boiling at neutral pH for several hours.

The nature of the receptor blockade was analyzed in several experiments. The binding was reversible and, as indicated in Fig 5, the blockade is apparently competitive since in this kind of plot the intersection with the ordinate remained the same in the presence of increasing amounts of the endogenous factor. We also found that the opiate receptor of the guinea pig ileum could be blocked by the factor (Fig 6). However, the factor was less effective on the ileum than on the SPM preparation, particularly after long term incubation.

### Discussion

The starting point for this investigation was the hypothesis that there might be an endogenous factor with affinity for the opiate receptor. There were earlier reports based on bioassays indicating the presence of such factors. Thus, Green and co-workers (Green *et al* 1963; Robinson *et al* 1967) described a lipoidal material which caused contraction of the isolated guinea pig ileum, probably by release of acetylcholine from endogenous stores. This effect was completely blocked by very low concentrations of morphine. When we followed the extraction scheme of Green and co-workers, we could not find any receptor blocking activity. Ungar and co-workers (Ungar and Cohen 1966; Ungar and Galvan 1968) have described the transfer of morphine tolerance by a low molecular weight compound from a morphine tolerant animal to a non-treated animal. Very recently, Hughes (1975) has found a factor in pig brain which is morphinomimetic on the mouse vas deferens and which is antagonized by naloxone. However, the complexity of biological assay systems of narcotics makes the interpretation of such experiments in terms of mechanisms of action difficult.

The present work shows a different approach to the problem. It is based on the hypothesis that the putative endogenous factor might bind to the same receptor site as opiate or affect their binding in an allosteric manner, thus allowing its presence to be measured by receptor blockade. We know from the work of several groups that the binding area of the opiate receptor is highly discriminative. All tested neurotransmitters have affinities which are magnitudes of order lower than those of the narcotics or their antagonists. Of a large number of

## Influences on Colonic and Small Intestinal Motility by the Cerebellar Fastigial Nucleus

By

JAN MARTNER

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### Abstract

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MARTNER J *Influences on colonic and small intestinal motility by the cerebellar fastigial nucleus* Acta physiol scand 1975 94 82-94

The fastigial influence on intestinal motility was investigated in acute experiments on chloralosed cats. Motility was recorded both from the small and large intestine. Electrical stimulation of the rostral fastigial pole produced in combination with a blood pressure rise increased motor activity in ileum and colon while jejunum could respond with either increased or decreased motility. The intestinal responses were neither secondary to changes in intestinal blood flow nor to baroreceptor reflexes induced by the increased blood pressure. The excitatory responses were not due to increased parasympathetic activity since sectioning of such pathways failed to abolish the responses. Instead interruption of adrenergic sympathetic discharge accomplished either by guanethidine or by sectioning of relevant nerves did eliminate the responses indicating that the fastigial effects were mediated by suppression of prevailing adrenergic tone. Noxious stimuli to the abdomen including laparotomy inhibit intestinal motility by a reflex increase in adrenergic discharge. It is suggested that fastigial influence on intestinal motility is mainly due to suppression of this reflex.

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Besides its well known control of somatomotor function the cerebellum has long been known to exert a widespread influence on autonomically innervated organ systems (cf Dow and Moruzzi 1958). Earlier experiments have mainly dealt with the cerebellar cortex while the interest has recently been focused on the cerebellar nuclei especially the fastigial nucleus. Thus electrical stimulation of its rostral pole elicits powerful pressor responses (Achari and Downman 1969; Miura and Reis 1969) which have been subject to further analysis in several studies (Achari and Downman 1970; Miura and Reis 1971; Lisander and Martner 1971, 1973; Achari, Al Ubaidy and Downman 1973). Recently it has been suggested that the fastigial pressor neurons are involved in the control of circulatory orthostatic reflexes (Doba and Reis 1972, 1974) but whether they are engaged in the control also of other autonomic functions is not known.

While this information is accumulating regarding cerebellar cardiovascular control much less is known of cerebellar influences on gastrointestinal motility. Inhibition of intestinal motility following stimulation of the vermis and the hemispheres has been reported (Voronin and Simkina 1938) and both inhibitory and excitatory gastrointestinal responses

have been observed on stimulation of various cerebellar parts including the fastigial nucleus (Beller and Talan 1971 Manchanda Tandon and Anuja 1972) Beller and Talan (1971) also claimed that rostral fastigial parts were effective in producing excitatory responses while caudal parts often produced inhibitory responses

Towards this general background it was considered of interest to investigate in more detail whether the fastigial pressor area besides its influence on cardiovascular control affects also other autonomically innervated organs where the present study was concentrated on the fastigial influence on spontaneous intestinal motility A preliminary report has been published (Lisander and Martner 1974)

## Methods

Experiments were performed on 50 cats of either sex which after induction with ether were anesthetized with chloralose 50–60 mg/kg. Free airways were secured by a tracheal cannula

### *Operative procedures and recordings*

Arterial pressure was recorded through a femoral catheter connected to a Statham P 73 AC transducer writing on a Grass polygraph while heart rate was measured by a Grass Tachograph unit. The secretion from the adrenals was eliminated in most animals by encircling ligatures and adrenalectomy. Substitution was given by i.v. injection of hydrocortisone (Solu-Gluc® Erco) 10 mg/kg. Intestinal motility was recorded in isolated 5 cm long small intestinal and colonic sections gently rinsed from their contents. For colonic isolation only loose ligatures were applied to avoid damage to the intramural nervous connections. Those parts of the small intestine that were not used for recording were extirpated together with the spleen and the greater omentum. After filling the intestinal loops with warm Tyrode solution motility was recorded as intraluminal volume changes transferred via a wide tube to a container of 5 cm diameter which was mounted on a Grass force-displacement transducer (FTOJ) for continuous recording of its weight. The level of the container was adjusted to obtain a constant intraluminal pressure of 5–10 cm H<sub>2</sub>O. In some cases small intestinal motility was instead recorded as pressure changes in intraluminal balloons.

The vagal nerves were dissected free in the neck and placed on ligatures so that they could be cut during the experiment. The sympathetic supply to the intestines along the superior mesenteric artery could be interrupted by cutting these nerves and also the lumbar colonic nerves were dissected free along the inferior mesenteric artery for subsequent cutting or for reversible blockade by cooling. Also the pelvic nerves were dissected free and placed on ligatures so that they could be cut in the course of the experiment. Intestino-intestinal inhibitory reflexes were elicited either by graded distension of a small isolated intestinal loop or by afferent stimulation of thin mesenteric pedicles.

Intestinal blood flow was recorded after heparinization as the outflow from the portal vein after ligation of all venous branches except those from the intestinal segment under study. After passing a closed optical drop recorder operating an ordinate writer the blood was returned via the femoral vein. For baroreceptor stimulation one carotid sinus region was isolated and perfused at various pulsating pressures with blood from one of the femoral arteries via a syngamotor pump. The contralateral sinus nerve was then cut as were the vagal nerves, thus eliminating other baroreceptor reflexes.

### *Cerebellar stimulations*

Topical cerebellar stimulations were induced by means of the Horsley-Clarke technique using a sharp (tip about 50–100 µm in diameter) monopolar stainless steel electrode insulated except for the tip. Square wave pulses of 1 ms duration were delivered by a constant current stimulator at intensities between 0.05 and 0 mA corresponding to a voltage range of 1.3 V and with frequencies between 10 and 50 Hz. The electrodes were inserted perpendicularly to the stereotaxic horizontal plane after trephination and partial removal of the tentorium.

### *Histological procedures*

After each experiment a small lesion was made around the electrode tip by diathermy at anodal current of 1 mA for 10 s. The head of the animal was perfused by saline followed by a 10% solution of formaldehyde. The

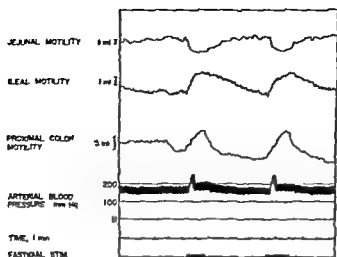


Fig 1 Cat 4.0 kg. Flaxedil 4 mg/kg i.v. artificial respiration Fastigial stimulation (50 Hz, 1 ms 0.1 mA) produces a marked pressor response while ileal and colonic motility is increased and jejunal motility inhibited

cerebellum and adjacent brainstem structures were then paraffin embedded sectioned in slices of 15  $\mu$ m and stained by the Nissl technique or in some cases by cresyl fast violet (Kluver-Barrera). In later experiments the cerebellum was frozen sectioned.

#### Drugs

Respiratory interferences with the motility recordings were sometimes impossible to avoid in which cases Gallamine iodide (Flaxedil<sup>®</sup>) 4 mg/kg b.wt. was given artificial respiration was then maintained by a respiratory pump. Pharmacological adrenergic blockade was induced by guanethidine (Ismelin<sup>®</sup> CIBA) 4 mg/kg b.wt. i.v. For  $\alpha$  blockade dibenzylamine (Smith Kline and French) 2-10 mg/kg b.wt. i.v. was used.  $\beta$  blockade was induced by propranolol (Inderal<sup>®</sup> ICI) 0.5-1 mg/kg b.wt. i.v. Atropine (atropine sulphate) was used in a dose of 0.5-1 mg/kg b.wt. i.v.

#### Results

Electrical stimulation of the fastigial nucleus affected small intestinal and colonic motility in almost all experiments. The response patterns most commonly found when all nervous influences were still intact are shown in Fig. 1 where fastigial stimulation elicits increased colonic and ileal motility while jejunal motility is inhibited. Sometimes however fastigial stimulation produced excitatory jejunal responses as well.

1 *Fastigial influence on jejunal motility* While the colon and ileum uniformly responded with increased motility upon fastigial stimulation the jejunum exhibited inhibitory responses in about half the experiments (11 cats) and excitatory responses in the other half (12 cats). In some cats both response patterns could be seen e.g. initial inhibitions which changed into excitatory responses later on, such a reversal occurring without any change of electrode position. During periods of jejunal inactivity increased motility was sometimes elicited as a rebound phenomenon upon cessation of the stimulation. The excitatory jejunal responses upon fastigial stimulation were not abolished by vagotomy (6 cats) but by cutting the adrenergic nerve supply or by guanethidine administration (4 cats).

The background of the jejunal inhibitions was more difficult to reveal since these respon

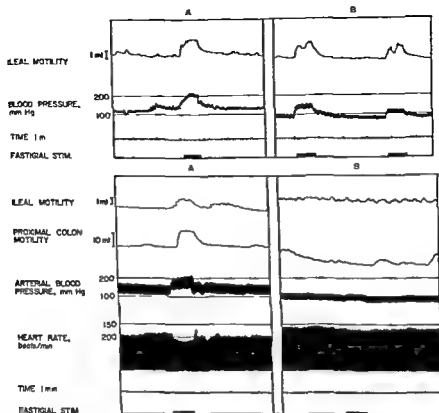


Fig. Upper section: Cat 24 kg. Effect of fastigial stimulations (50 Hz, 1 ms, 0.1 mA) before (panel A) and after (panel B) bilateral vagotomy. Note that the prompt ileal excitatory response remains after vagotomy.

Lower section: Cat 30 kg. fastigial stimulation (50 Hz, 1 ms, 0.04 mA). Between panels A and B guanethidine 4 mg/kg bwt i.v. has been given. The fastigially induced excitatory intestinal responses in A are abolished in B while both the colon and ileum now exhibit spontaneous motility.

ses were not as reproducible as the excitatory ones but they appeared to require intact adrenergic mechanisms while they persisted after vagotomy.

**2. Fastigial influences on ileal motility.** In virtually all cats (39 out of 42) the fastigial pressor response was associated with a prompt increase of ileal motility and inhibitions were never observed. The ileal motor responses were often very powerful and direct inspections sometimes showed intensely contracted even blanched ileal segments. Analysis of the pathways conveying these excitatory ileal responses revealed that the vagal nerves were not necessary (upper section of Fig. 2, A and B before and after vagotomy). Thus cutting the vagal nerves during the experiment in no way eliminated the motility increase to fastigial stimulation though the response was in a few cases somewhat reduced. On the other hand, sectioning of the adrenergic supply eliminated the ileal excitatory responses (3 cats) whether the vagal nerves were cut or not as did administration of guanethidine (7 cats) illustrated in Fig. 2 lower section. The effect of  $\alpha$ -adrenergic blocking agents was then more variable.

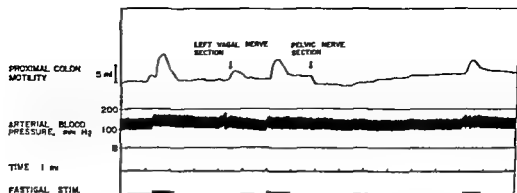


Fig. 3 Cat 2.2 kg, right vagal nerve cut. Fastigial stimulation (50 Hz, 1 ms, 0.2 mA) elicits powerful colonic contraction which persists after complete vagotomy and also after bilateral pelvic nerve section, though somewhat reduced in extent.

in some cats the response vanished but in others it persisted although now appearing first after a delay of some 30 s

**3 Fastigial influence on colonic motility** The proximal colon responded in 37 out of 40 cats with contractions upon fastigial pressor stimulation as did middle parts of the colon. These contractions usually occurred with few seconds delay and persisted throughout the 30–60 s stimulation period to cease promptly when stimulation was discontinued.

The distal colon yielded more variable results with no responses in 2 cats and with weak and delayed contractions in three cats. These responses were however not due to peristalsis conducted distally from the proximal colon as they could still be obtained when the continuity of the gut was broken. Often these colonic responses became gradually less pronounced in the course of the experiment which was hardly due to any cerebellar damage around the electrode since the pressor response never decreased.

The mediation of the colonic excitatory responses to fastigial stimulation was analysed either by consecutive sectioning of the peripheral nerves or by using pharmacological blocking agents. The effect of sectioning the cholinergic pathways were analysed in 20 cats. Fig. 3 illustrates such an experiment. Cutting the left vagal nerve (the right one had been earlier cut) slightly reduced but did not abolish the colonic response. If also the pelvic nerves were cut, the response was further reduced but not abolished—a persistent finding in all these experiments. Cholinergic blockade with atropine (1 mg/kg *li wt* *i.v.*) considerably reduced and delayed the motility increases to fastigial stimulation but did in most cases not abolish them (Fig. 4 panel B). Pelvic nerve sectioning did not even now abolish the response while guanethidine did so (panel C).

The adrenergic pathways are mainly in the lumbar colonic nerves supplying the entire colon, while the proximal colon receives adrenergic innervation also from the thoracic sympathetic outflow via the splanchnic nerves (cf. Hultén 1969). Interpretation of the results after adrenergic blockade is difficult because this procedure always brings about a marked increase in spontaneous colonic motility which might mask the effect of fastigial stimulation. However, after sectioning or cooling the lumbar colonic nerves no clearcut colonic respon

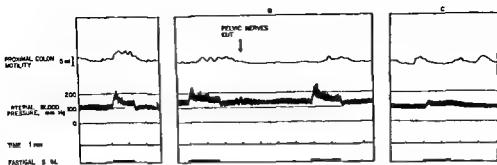


Fig 4 Cat 2.5 kg vagal nerves cut. A Prompt colonic excitation elicited by fastigial stimulation (50 Hz, 1 ms 0.2 mA) B Colonic response after atropine 1 mg/kg between the first and second stimulations the pelvic nerves are cut, which reduces and delays the response C Guanethidine 4 mg/kg, increases spontaneous colonic motility but eliminates response to fastigial stimulation

ses could ever be recorded upon fastigial stimulation (9 cats). If the thoracic sympathetic supply was interrupted by cutting the nerves along the superior mesenteric artery the responses were sometimes reduced but could still be obtained. The adrenergic blocking drug guanethidine also increased the basal colonic motility but abolished in all 14 cats so treated the earlier very powerful contractions upon fastigial stimulation (Fig 2 lower section).

The  $\alpha$  blocking agent dibenzylne (4 mg/kg) rendered the colon more prone to contract spontaneously but sometimes failed to eliminate the colonic responses to fastigial stimulation. If dibenzylne was combined with a  $\beta$  adrenergic blocking agent (propranolol 0.5–1 mg/kg bwt i.v.) the colonic responses were further reduced but not always completely eliminated.

**4 Suppression of motility by fastigially induced adrenal catecholamine release** The adrenals were as mentioned excluded in most experiments by ligation to eliminate the effects of circulating catecholamines liberated by fastigial stimulation (Lisander and Martner 1973). If however the adrenal glands were left intact and guanethidine given to eliminate the neurogenic adrenergic influences on intestinal motility fastigial stimulation induced long lasting inhibitory effects in all intestinal segments studied (Fig 5). The delay in onset and the longlasting responses with regard to intestinal motility, blood flow and heart rate strongly speaks in favour of a hormonal mechanism. It is known that guanethidine does not affect the medullary release of catecholamines but rather enhances their effects (Abercrombie and Davies 1963).

**5 Are the motility responses due to intestinal blood flow changes or to baroreceptor reflexes?** To exclude that the fastigial influence on intestinal motility was secondary to changes in blood flow during stimulation arrangements were made in 4 experiments for simultaneous recordings of intestinal motility and blood flow. Although there was a substantial resistance increase in the intestinal vascular bed during fastigial stimulation intestinal blood flow was only slightly affected because of the concomitant pressure rise. Independent of the slight changes of blood flow increased motility was constantly elicited by fastigial pressor area stimulation.

If a blood pressure rise of same magnitude as that induced by fastigial pressor area



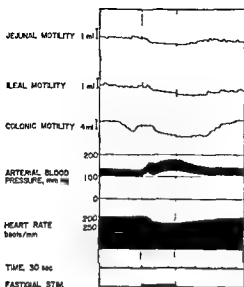


Fig 5 Cat 2.7 kg intact adrenals but guanethidine 4 mg/kg given. Note the inhibition of motility in all intestinal parts upon fastigial stimulation. The delayed onset and longlasting effects of the cardiovascular and intestinal responses point towards a hormonal mechanism. The small regular oscillations seen in the motility recordings reflect increased respiratory movements sometimes obtained by fastigial stimulation.

stimulation was caused by either baroreceptor unloading or by rapid dextrane-Tyrodé infusion these procedures would reflexly decrease or increase respectively intestinal blood flow. The procedures did not however affect intestinal motility. The possibility that enhanced intestinal motility during fastigial stimulation was due to a reflexly reduced adrenergic tone evoked via the baroreceptors upon the fastigial pressor response was excluded by the type of experiment illustrated in Fig 6. When the isolated carotid sinus region was exposed to an increased pressure no change in ileal motility was seen while fastigial stimulation performed during this period of intensified baroreceptor activation still enhanced ileal motility.

**6. Fastigial suppression of the intestino-intestinal inhibitory reflex.** Distention of a gastrointestinal segment, stimulation of afferent mesenteric fibres or noxious abdominal stimuli produce prompt adrenergic inhibition of gastrointestinal motility. Procedures here included

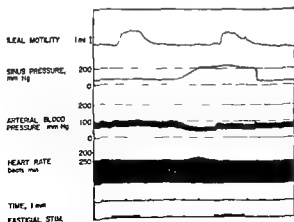


Fig 6 Cat 2.6 kg both vagi and right carotid sinus nerve cut. Left carotid sinus prepared for pressure perfusion. Fastigial stimulation (50 Hz, 1 ms, 0.2 mA) elicits similar ileal excitatory responses whether the right sinus region is exposed to systemic pressure (left) or high constant pressure (right) which causes a reflex arterial pressure reduction and bradycardia.

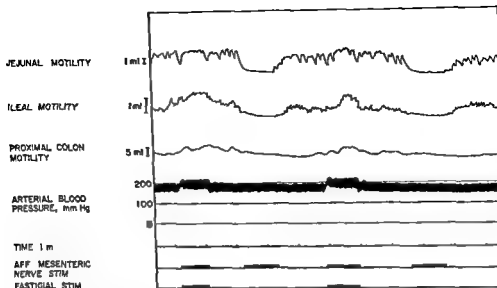


Fig 7 Cat 2.0 kg vagal nerves cut. Afferent mesenteric nerve stimulation (5 Hz, 1 ms 7 V) induces inhibitions of spontaneous intestinal motility but this reflex inhibitory response is blocked by concomitant fastigial stimulation

in the terminal intestino-intestinal inhibitory reflexes (*cf* Youmans 1968). Fig 7 shows such a reflex intestinal inhibition caused by afferent mesenteric nerve stimulation. When a simultaneous fastigial stimulation is performed these reflex inhibitions can often be completely blocked. Such fastigial suppressions of intestinal reflex inhibition could be demonstrated both in the small intestine and in the colon although the colonic effects were more variable and could not always be completely suppressed.

**7 Histological examination of the fastigial points stimulated.** The fastigial pressor area is located in ventro-medial parts of the fastigial rostral pole (Miura and Reis 1969). The fastigial parts affecting the intestinal tract correspond quite well with those producing pressor responses as illustrated in Fig 8. Thus virtually all stimulation points yielding intestinal responses also caused pressor responses and were gathered in ventro-medial parts of the fastigial rostral pole and in the adjacent ventro-rostral cerebellar white matter. Effective stimulation points were plotted on maps schematically drawn from frontal sections corresponding to the coordinates P 8.0-P 9.0 given by Snider and Niemer (1961). In Fig 8 the small intestinal responses are shown on the left side of each drawing while the colonic ones are shown on the right side. Virtually all responsive points were combined with generally marked pressor responses. The likelihood of inducing intestinal responses increased with the intensity of the pressor responses although a marked pressor response was not throughout a prerequisite as can be seen in Fig 3 and 6 where only small or moderate blood pressure increases are recorded. The points in Fig 8 representing effective motility responses in the various intestinal segments seem to be intermingled and localized in or close to the ventro-medial border of the rostral fastigial parts.

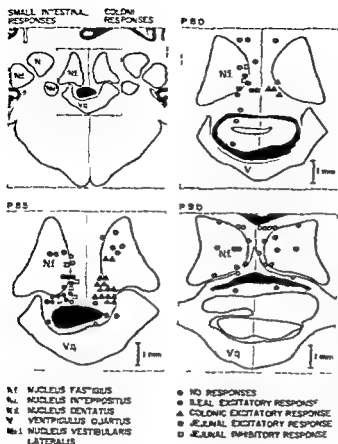


Fig. 8 Frontal section through cerebellum and brainstem. The area within the hatched lines seen in the upper left drawing is magnified and in the subsequent drawings shown at three different levels 8.85 and 9 mm posterior to the interaural line. The colonic responses are marked on the right side while the small intestinal responses are shown on the left side of each figure. Explanation of signs and abbreviations are shown in the figure. For further information see text.

### Discussion

The present study shows that fastigial stimulation can influence the "spontaneous" motility in the jejunum, ileum and colon. The responses displayed regional differences, with uniformly excitatory responses in the colon and the ileum in the prevailing experimental situations, while the jejunum could show both inhibition and excitation. The neurogenic excitatory colonic and ileal responses, elicited by fastigial stimulation, can theoretically be achieved by an increased parasympathetic activity or/and by a reduced sympathetic tone. Here the situation for the colon is particularly complex as it receives parasympathetic cholinergic innervation from the vagal nerves, which convey excitatory impulses restricted to proximal parts, while the sacral parasympathetic outflow supplies the entire colon via the pelvic nerves. Likewise, the adrenergic innervation is divided into a rostral division, supplying the proximal colon via the splanchnic nerves while the lumbar colonic nerves innervate largely the entire colon (cf Hultén 1969 Rostad 1973).

In general, sectioning of the cholinergic supply could be performed without abolishing the colonic and ileal excitatory responses, indicating that they were conveyed by a suppression of an adrenergic influence. Accordingly cutting the mentioned sympathetic nerves, or administration of guanethidine, abolished the excitatory responses to fastigial stimulation.

The fact that atropine markedly reduced and occasionally abolished these excitatory responses by no means speaks against the involvement of primarily adrenergic mechanisms. The reason is that adrenergic inhibitory influence on gastrointestinal motility is generally exerted at the intramural ganglionic level and thus calls for a prevailing activity in the intramural excitatory cholinergic neurons to be revealed (Norberg 1964, Kewenter 1965, Jansson and Martinsson 1966, Hultén 1969). Atropine will eliminate their excitatory influence on the intestinal smooth muscles but not necessarily a sectioning of the preganglionic cholinergic fibres as long as an intrinsic activity is maintained in the intramural cholinergic plexa. Therefore in the latter case a central suppression of a prevailing adrenergic inhibitory influence will reveal itself as an excitatory influence on intestinal motility. Thus the cerebellum in general exerts its main effects on spontaneous intestinal motility by decreasing adrenergic activity via the fastigial nuclei while the cerebellar cortex in turn is known to exert a generally inhibitory influence on fastigial activity.

It is generally assumed that the adrenergic inhibitory influence at the ganglionic level is exerted by  $\alpha$  receptor mechanisms (cf Furness and Costa 1974) but even large doses of dibenzylamine or/and phentolamine did not always abolish the fastigially induced intestinal contractions although they were now more delayed in onset at the same time as the intestine exhibited a markedly increased spontaneous motility. This remaining excitatory response after complete nerve blockade could still be detected after cutting the pelvic and vagal nerves. However some cholinergic excitatory fibres appear to reach the colon via its sympathetic supply (Varagic 1956, Hultén 1969) and possibly such cholinergic fibres may have been activated by fastigial stimulation in analogy to the finding that the fastigial nucleus can modify e.g. the cardiac vagal nerve activity (Lisander and Martner 1971, Achari *et al* 1973). Whatever the case the suppression of the adrenergic fibre activity to the gastrointestinal tract appears to be the most prominent excitatory influence on gastrointestinal activity exerted by the fastigial neurons.

There seems to be a good correlation between the fastigial pressor response and the fastigial influence on intestinal motility, powerful pressor responses being regularly associated with pronounced intestinal excitatory responses and *vice versa*. As shown in Fig. 8 the effective stimulation points are clustered around the rostral ventro-medial fastigial pole and just ventromedially to the nucleus, presumably then representing the efferent pathways from the nucleus. The majority of the neurons in rostral fastigial parts send their axons ventromedially and as crossed fibres giving rise to the hook bundle (Matsushita and Iwahori 1971).

No clearcut topographical fastigial organisation could be traced concerning responses in different gastrointestinal parts, instead effective stimulation points usually induced responses throughout the intestinal tract. However it should be realized that the current spread might amount to  $\approx 5$  mm (Wise 1972) and that locating the electrode may have an error of up to 0.5 mm due to the size of the lesion induced.

The close association between cardiovascular pressor responses and intestinal responses to fastigial stimulation might imply that the latter were a consequence of the former being for example secondary to neurogenic changes in intestinal blood flow. Thus mechanical reductions of intestinal blood flow can inhibit intestinal motility though first when the

reduction in flow is quite marked (Celander 1959 Kock 1959). To exclude any such interactions intestinal blood flow and motility were recorded simultaneously in some experiments revealing that the changes in blood flow secondary to fastigial stimulation were quite small. It is therefore unlikely that e.g., the jejunal inhibitory responses to fastigial stimulation should be secondary to any reduced jejunal blood flow. Furthermore in the colon and ileum the motility responses were excitatory making it still more unlikely that they were secondary to changes in blood flow. Finally the motility responses were usually prompt in onset, making it highly unlikely that they should be secondary to the gradual change in local chemical environment induced by the neurogenic restriction in blood supply.

Intestinal motility may be influenced by adrenal catecholamine release induced by fastigial stimulation. When the adrenals were left intact and guanethidine was given to block the neurogenic intestinal responses delayed but longlasting motility inhibitions were obtained as a result of the catecholamine release. This illustrates the importance of eliminating the adrenals in studies of neurogenically mediated influences from the cerebellum on gastrointestinal motility. In the vast majority of the present experiments the adrenal glands were however excluded in which situation the jejunal inhibition must be ascribed to nervous mechanisms.

Finally the possibility remains that the intestinal responses were secondary to baroreceptor reflex influences induced by the fastigial pressor responses. Such reflexes might, for example suppress the sympathetic discharge to the intestine. However such a mode of influence was excluded by the fact that even intense and constant baroreceptor activations did not influence the changes in intestinal motility elicited by fastigial stimulation.

It is highly unlikely that the cerebellum has any direct nervous connections with the ly controlled organ systems and its influence is probably rather one of modifying the reflex arcs controlling visceral functions. Consequently the fastigial responses would be dependent on a prevailing activity in such arcs which, in turn is likely to vary considerably with the experimental situation. This may explain the varying jejunal responses to fastigial stimulation: sometimes changing from inhibition to excitation in the course of the same experiment. The dependence on the experimental situation has a bearing also on comparisons of results in different studies of cerebellar autonomic function. In the present study the experimental procedures certainly tend to elicit the spinally mediated *intestino-intestinal inhibitory reflex* by means of the inevitable abdominal surgery inducing sympathetic discharge to the entire gastrointestinal tract. As discussed above the fastigial nucleus appears to exert its main control of intestinal motility by modulating adrenergic tone where the preferential influence in the present situation is one of suppression. The spinal *intestino-intestinal reflex* is subject to control from higher CNS levels and can for example be efficiently suppressed from the medullary "depressor area" (Johansson Jonsson and Ljung 1965 1968).

Besides by operative trauma, the *intestino-intestinal inhibitory reflex* can be elicited also by electrical stimulation of mesenteric afferents or by intestinal distension procedures which can be graded and, if strong enough are able to entirely inhibit intestinal motility. In case the fastigial nucleus is effective in suppressing the *intestino-intestinal reflex*, fastigial stimulation would be expected to block the reflex inhibition induced by e.g. afferent mesen-

teric stimulation which was indeed the case (Fig 7). If however supramaximal afferent stimulation was applied for eliciting the reflex it was increasingly difficult to block it by fastigial stimulation.

A regional difference in sympathetic control of the small intestine has been described by Kewenter (1965) with a preponderant inhibitory influence on the ileum. This may explain why the jejunal and ileal responses to fastigial stimulation were often different. As fastigial stimulation seems to uniformly suppress sympathetic activity to the gastrointestinal tract it would be expected that the resulting increases in motility would be particularly pronounced in those sections of the small intestine where the adrenergic inhibitory influence was strongest to start with. Such a view corresponds well with the response pattern generally found in the small intestine.

In conclusion the fastigial nucleus and hence the cerebellum is involved in the autonomic regulation of intestinal motility where one important mode of action appears to be a modulation of adrenergic inhibitory discharge. A possible mechanism for this might be fastigial suppression of the spinal intestino-intestinal inhibitory reflex which is conveyed via adrenergic fibre suppression of the activity in the intramural cholinergic ganglion cells. Indeed results from recent experiments to be reported in a subsequent paper strongly support such a mechanism. This by no means denies the possibility that cerebellar control of intestinal autonomic regulation may be exerted also by other mechanisms that might be revealed during other experimental conditions.

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## Influences on the Defecation and Micturition Reflexes by the Cerebellar Fastigial Nucleus

By

JAN MARTNER

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### Abstract

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The influence of the cerebellar fastigial nucleus on the defecation and micturition reflexes was investigated in chloralosed cats with recordings of colonic blood flow and motility intravesical and intra-abdominal pressures. Whenever effective topical fastigial stimulation regularly suppressed both somatomotor and autonomic components of the defecation reflex to the extent that the straining movements as well as the colonic vasodilator and motor responses associated with defecation could be completely inhibited. Bladder motility could either be suppressed or enhanced depending both on prevailing bladder tone and on the fastigial site stimulated. The autonomically conveyed inhibitory responses were in both cases independent of the adrenergic sympathetic pathways since they were unaffected both by sympathetic nerve sectioning and by adrenergic blocking drugs but eliminated by pelvic nerve section. It is suggested that the mentioned fastigial inhibitory influences are exerted on the spinal parasympathetic reflexes controlling the bladder and colon. Parallels between fastigial control of autonomic and somatomotor mechanisms are discussed.

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Increasing evidence is accumulating in support of a role for the cerebellum also in autonomic control. Especially interesting in this context is the fastigial nucleus which is very potent in eliciting both cardiovascular (Achari and Downman 1969; Miura and Reis 1969) and gastrointestinal responses (Lisander and Martner 1974) upon electrical stimulation. Both types of responses are induced from the rostral ventromedial fastigial pole and involve predominantly a modulation of adrenergic sympathetic discharge which is enhanced to the circulatory system but as has been recently shown is generally suppressed to the colon and the small intestine (Martner 1975). It was considered of interest to investigate whether also parasympathetically conveyed autonomic responses can be directly influenced from the fastigial nucleus where especially the defecation and micturition reflexes are of relevance. To fully reveal the fastigial influence on the parasympathetically induced colonic motor responses during defecation it was necessary to eliminate direct fastigial adrenergic modulation of the colon (Martner 1975).

A possible fastigial influence on the defecation reflex is of particular interest also from another point of view insofar as it involves easily elicited somatomotor and autonomic components. The former requires for its full expression supraspinal levels while the la



■ basically conveyed at spinal sacral levels (Garry 1933) though being considerably influenced also by bulbar and suprabulbar autonomic centres (Hatcher and Weiss 1923 Koppanyi 1930 Hess and Brugger 1943)

While the cerebellar control of somatomotor mechanisms ■ by now fairly well understood comparatively little is known about the principles of cerebellar autonomic control. Therefore investigations of cerebellar effects on reflexes that involve both somatomotor and autonomic components might help to elucidate how the cerebellum influences autonomic mechanisms. For such reasons the first part of the present study deals with the fastigial influences of the somatomotor and autonomic components of the defecation reflex.

Concerning the urinary bladder several authors have reported cerebellar excitatory as well as inhibitory influences especially from the vermal cortex (Whiteside and Guyton 1952 Emerson *et al* 1961 Bruhn *et al* 1961 Rasheed Manchanda and Anand 1970). Also from the fastigial nucleus both micturition (Chambers 1947) and suppression of the micturition reflex (Bradley and Teague 1969) can be elicited and bladder tone can be either increased or decreased (Bellar and Talan 1971 Manchanda and Bhattarai 1972 Lisander and Martner 1974). To further characterize the fastigial structures influencing autonomic mechanisms it was considered of interest to investigate whether they influence also bladder motility in a more regular and specific way.

A brief preliminary report covering part of the present results has earlier been presented (Lisander and Martner 1974).

### Methods

Experiments were performed on 30 cats anesthetized by chloralose 50–60 mg/kg b.wt. Free airways were secured by a tracheal cannula.

**Stimulations.** For cerebellar stimulation the Horsley Clarke technique was utilized exploring the fastigial nucleus by electrodes inserted perpendicularly to the horizontal plane. Stimulation was performed via thin monopolar stainless steel electrodes insulated except for the tip (about 50–100  $\mu$ m in diameter) using square wave pulses of 1 ms from a constant current stimulator. The intensities used were varied between 0.03 and 0.3 mA (corresponding to a voltage range from 1 to 3.5 V) at a frequency range of 10 to 50 Hz. After each experiment a small lesion was induced around the electrode tip by an anodal current of 1 mA for 10 s. The head of the animal was then perfused by saline followed by a 10% solution of formaldehyde. Relevant cerebellar parts were frozen sectioned and the stimulation points identified.

**Recordings.** Arterial pressure was recorded through a femoral catheter connected to a Statham P 23 AC transducer writing on a Grass polygraph. Heart rate was measured by connecting the pressure recording amplifier to a Grass Tachograph unit. When bladder pressure was recorded the abdominal wall overlying the urethra was opened by a small incision and a catheter inserted into the bladder via the urethra. Variations in intravesical pressure reflecting bladder motility were then recorded by connecting the catheter to a transducer. A saline reservoir adjustable to various levels was connected to the catheter via a side branch thus allowing for bladder filling at different pressures. Sometimes the abdominal incision was extended so that the hypogastric and pelvic nerves could be isolated placed on ligatures and cut in the course of the experiment.

The defecation reflex was elicited either by afferent stimulation of one cut pelvic nerve the other being left intact or by applying a mechanical fractional stimulation to the rectal mucosa (J. Hultén 1969). For recording colonic motility and blood flow the abdominal wall was opened by a midline incision and the small intestine was extirpated together with the spleen and greater omentum. About 5 cm of the most proximal colonic loop was isolated gently rinsed from contents and filled with Tyrode solution. The loop was distally isolated by loose ligatures to avoid damage of the intramural nervous connections. Colonic motility was recorded as intraluminal volume changes transmitted via a wide tube to a container with a diameter of 5 cm mounted on a Grass force displacement transducer FTO 3 thus continuously recording the weight of the container. The level of the container was adjusted to obtain an intraluminal

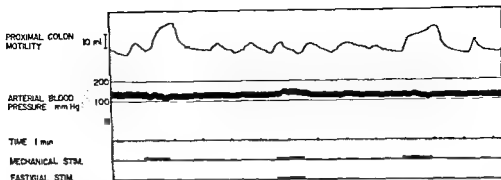


Fig 1 Cat 3.0 kg. Adrenergic fibres blocked by guanethidine 4 mg/kg. Mechanical rectal stimulation elicits powerful colonic contractions superimposed on spontaneous motility. Note that a concomitant fastigial stimulation (50 Hz, 1 ms, 0.04 mA) completely abolishes the colonic motor responses.

pressure of 5–10 cm  $H_2O$ . Colonic blood flow was determined in heparinized animals by recording the venous outflow from the superior mesenteric vein after extirpation of the small intestine. After passing a closed optical drop recorder operating an ordinate writer the blood was returned to the animal via the femoral vein.

**Drugs.** Since respiratory movements sometimes interfered with the colonic events gallamine iodide (Flaxedil®) 4 mg/kg b.wt. i.v. was in these cases given to avoid such disturbances, whereupon artificial respiration was given. No muscle paralyzing drugs were however given when the straining movements in connection with defecation were to be recorded; these were registered as pressure increases in an intraabdominal balloon. In most experiments the secretion from the adrenals was eliminated by encircling ligatures around both glands and adrenocortical substitution was then given by i.v. injection of hydrocortisone (Solu-Gluc® Eeco) 10 mg/kg b.wt. In some experiments the lumbar colonic nerves were dissected free from the inferior mesenteric artery and cut distally to the ganglion but in most cases the adrenergic supply to the colon was instead blocked by guanethidine (Ismelin® CIBA) 4 mg/kg b.wt. i.v. sometimes in combination with dibenzylamine (Smith Kline and French) 4 mg/kg b.wt. i.v. As an  $\alpha$ -blocking agent phentolamine (Regitin® CIBA) 1 mg/kg b.wt. i.v. was also used.  $\beta$ -blockade was induced by propranolol (Inderal® ICI) 0.5–1 mg/kg b.wt. i.v. Atropine (atropine sulphate) was used in a dose of 0.5–1 mg/kg b.wt. i.v.

## Results

### 1 Fastigial influence on defecation

Defecation involves activation of both somatomotor and autonomic reflex arcs where straining movements increase the intraabdominal pressure and together with colonic contractions help to expel the faeces. Colonic vasodilatation also forms part of the pattern (Hultén 1969). All these reflexly induced changes were found to be influenced by fastigial stimulation. As illustrated in Fig. 1 the reflex colonic contractions elicited by mechanical rectal stimulation could be completely suppressed in the adrenergically blocked animals. This was the case in 7 cats and only in 2 experiments fastigial stimulation failed to attenuate the colonic response. Likewise reflex colonic contractions induced by afferent pelvic nerve stimulation were inhibited as well (2 cats). Atropine reduced but did not eliminate the reflex colonic contractions and the small remaining reflex motor responses to rectal stimulation were also suppressed by fastigial stimulation.

Fig. 2 shows that the straining movements reflexly induced by afferent pelvic nerve stimulation could be completely suppressed by fastigial stimulation. Mechanical rectal

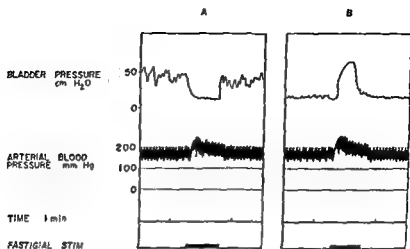


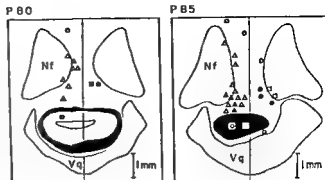
Fig. 5 Cat 2.8 kg. Fastigial stimulation (50 Hz 1 ms 0.1 mA) induces inhibition of bladder motility when performed against a background of high sustained intravesical pressure (panel A). The same fastigial site elicits bladder contraction if stimulation is performed during a background of low bladder tone. The small oscillations in bladder pressure seen during bladder inhibition are due to respiratory movements.

fastigial influence could now be traced. Atropine (1 mg/kg i.v.) substantially reduced the reflex bladder contractions but fastigial stimulation suppressed also the small remaining contraction waves.

Bladder contractions could also result upon fastigial stimulation although they were not so easily demonstrated as were the bladder inhibitions. However, once appearing they could be quite prominent (Fig. 5 panel B). This figure further illustrates that the very same fastigial point from which excitatory responses could be elicited (panel B) could also depress bladder motility once a pronounced and sustained reflex bladder tone was at hand (panel A). Neither the excitatory nor the inhibitory bladder response were strictly confined to the fastigial pressor area although the former type of response was usually associated with the pressor response.

The left side of Fig. 6 summarizes the points from which bladder responses were elicited. The fastigial nucleus can evidently not be divided into one excitatory and one inhibitory area in this very respect, even though the bladder inhibitory responses (open triangles) tend to cover a larger area. Contrary to most other fastigial autonomic effects including bladder excitation, the inhibitory bladder response seems to follow an all or nothing law. Thus while low stimulation frequencies did not noticeably influence bladder tone, a gradual frequency increase suddenly induced total suppression of bladder contraction, usually when 10–20 Hz were reached. This frequency range corresponds well with that where fastigial influences on *e.g.* blood pressure and gastrointestinal motility become obvious (Lisander and Martner 1975). However, the latter type of responses, essentially dependent of discharge changes in the sympathetic adrenergic fibres, are characterized by a gradually rising frequency-response curve which reaches a plateau at 40–50 Hz, while the parasympathetically mediated bladder inhibitory responses, once appearing, were then always maximal, *i.e.* producing 100 per cent suppression of the bladder motility.

Fig 6 Cerebellar frontal sections 8 and 8.5 mm posterior to the interaural line. The bladder responses are shown to the left and the defecation responses to the right. Open circles: No response. Open triangles: Inhibitory bladder responses. Filled triangles: Excitatory bladder responses. Filled dots: Suppression of colonic motor responses. Filled squares: Suppression of colonic vasodilatation. Open squares: Suppression of straining movements. Nf: Nucleus fastigii. Vq: Ventriculus quartus.



### Discussion

Colonic motility elicited from the rectum includes profound reflex contractions particularly of the proximal but also of the distal colon (Schmidt and Fulgraff 1963) where both afferent and efferent pathways run in the pelvic nerves (Hultén 1969). The defecation reflex can be elicited either by mechanical rectal stimulation or by pelvic afferent stimulation. In both cases fastigial stimulation could completely abolish the colonic motor response, showing that the fastigial influence cannot be a matter of *e.g.* any neurogenically conveyed suppression of receptor sensitivity in the rectal mucosa. Hypothalamic stimulation can inhibit spontaneous colonic motility (Wang *et al.* 1940; Sheehan 1942; Rostad 1973) and the latter investigator found that this inhibitory effect was abolished by adrenergic blockade. In contrast the fastigially induced suppressions of reflex colonic motility reported here were due to a direct inhibition of the parasympathetically mediated defecation reflex and were not a matter of any activation of colonic sympathetic fibres, obvious from the fact that all animals used were subject either to pharmacological adrenergic fibre blockade or to section of the lumbar colonic nerves. This procedure was also necessary for the full expression of the parasympathetically mediated reflex colonic motor and vasodilator responses which are known to be suppressed by a prevailing sympathetic discharge, presumably at the intramural ganglionic level (Hultén 1969). Moreover the fastigial influence on spontaneous colonic motility in cats is excitatory in nature and mediated by a suppression of prevailing sympathetic discharge (Lisander and Martner 1974). Elimination of the sympathetic inhibitory influence on parasympathetically mediated colonic responses which would certainly interfere with the present type of study was another reason for using various kinds of adrenergic blockade. Ligation of the adrenals excluded the possibility that the fastigial inhibition of the reflex colonic motility was mediated by a release of catecholamines from the adrenal medulla (Lisander and Martner 1973).

Besides influencing colonic motor activity, fastigial stimulation could also completely suppress the reflex vasodilatation accompanying defecation. Efferent pelvic nerve stimulation elicits mucosal vasodilatation and secretion (Wright, Florey and Jennings 1938) and Hultén (1969) showed that the vasodilatation forms part of the defecation reflex and is neither eliminated by adrenergic nor by cholinergic blocking agents. Whichever the trans-

mitter mechanism involved at these parasympathetic nerve endings the fastigial nucleus could completely suppress this vascular response

Interestingly the somatomotor component of the defecation reflex was affected by the same fastigial area and in the same inhibitory way as the autonomic components. Thus total suppression of the straining movements was always induced by fastigial stimulation. Cerebellar modulations of somatomotor mechanisms are wellknown ever since Lowenthal and Horsley (1897) reported inhibition of decerebrate rigidity by cerebellar stimulation. Since the fastigial nucleus always modified the somatomotor and autonomic components of the defecation reflex in the same direction it seems likely that cerebellar control may be exerted according to similar principles both concerning somatomotor and autonomic mechanisms.

In fact other parallels between the cerebellar influence on somatomotor and autonomic mechanisms do exist and can be illustrated *e.g.* by comparing wellknown cerebellar somatomotor effects with the results obtained on bladder autonomic functions. It was found in the present experiments that the fastigial nucleus could both inhibit and augment bladder motility in consonance with the findings of Beller and Talan (1971) and Manchanda and Bhattarai (1972). Determinant of the direction of the response was both the prevailing bladder tone and the cerebellar site stimulated. Thus moving the electrode only 0.5 mm sometimes changed an inhibitory response into an excitatory one. Such a reversal could be obtained even from the same fastigial site: inhibitions occurring during periods of reflexly induced bladder contractions but changing into excitatory responses when the contractions had spontaneously subsided. Similar effects have been induced from the cerebellar cortex (Whiteside and Guyton 1952). The same kind of variable cerebellar influence *de* on background activity has been illustrated also concerning the cardiovascular system (Moruzzi 1938) and is also demonstrated concerning somatomotor reflexes (Moruzzi 1936). Thus cerebellar stimulation produced inhibition of extensor hypertonia in decerebrate cats while increased extensor tone was occasionally recorded if stimulation was undertaken during hypotonia sometimes as a rebound effect at the end of the stimulation. Such rebound phenomena have been observed both for the autonomic and somatomotor components of the defence reaction during sham rage (Zanchetti and Zoccolini 1954) and were also observed concerning the purely autonomic responses of the bladder in the present study.

The finding that cerebellar stimulation produced a constant and generalized inhibition of *e.g.* the defecation reflex in the present experiments does however not necessarily imply that the cerebellum normally always exerts a suppressive influence in these respects. Electrical stimulation of specific brain areas does not necessarily simulate *in vivo* patterns in a precise way. For example such stimulations excite certainly also antidromically and without discrimination both afferent and efferent pathways and may affect both fibres and neuron somata. Hence if effects can be demonstrated in the form of fastigial suppression of defecation and micturition as in this case it is no doubt indicates that the cerebellum is involved in the control of these reflexes but reveals little about the way by which it is normally brought about whether concerning levels of influence intraspinal pathways *etc.* Thus if topical electrical stimulations are applied somewhere in the complex cerebellar

machinery stereotype response patterns are likely to be obtained e.g. in the form of over all inhibitions while the normal cerebellar coordination might well involve both inhibitory and facilitatory modulations depending on the prevailing input of information. It is known that the cerebellum receives afferent projections also from visceral organs (Widén 1955).

From the present data alone it is therefore not possible to decide on what level the cerebellum expresses its autonomic influence. It is however most unlikely that there are specific cerebellar projections to the effector organs studied rather the results strongly indicate that the cerebellum modifies the spinal autonomic reflex arcs involved at the bulbar or/and spinal levels. As for the micturition reflex the cerebellar interaction might take place in the bulbar reticular formation from which descending pathways influence the sacrospinal micturition center. A suppression of the parasympathetic micturition reflex following fastigial stimulation might however *a priori* also be due to sympathetic activation to the bladder perhaps then occurring as a parallel to the intensified sympathetic drive on the circulatory system that constitutes the fastigial pressor response. The bladder is *via* the hypogastric nerve supplied with sympathetic fibres that can induce bladder relaxation (Elliott 1907). In the present study however neither pharmacological adrenergic fibre blockade nor sympathetic fibre section affected the fastigially induced depression of bladder motility. Thus the bladder inhibitions obtained in the present experiments could not be a consequence of any sympathetic activation. Instead the cerebellar influence studied here was in all likelihood due to a suppression of the parasympathetically mediated micturition reflex *per se* exerted either at the bulbar or spinal levels as suggested also by Bradley and Teague (1969). Indeed the fastigial nucleus projects to the bulb and as can be seen from the drawing in Fig. 6 responses were also obtained from points along the fastigio-bulbar pathways.

Although the bladder responses were not exclusively elicited from the fastigial pressor area (Fig. 6) which is restricted to the rostral ventromedial fastigial pole this area proved to be effective in producing both excitatory and inhibitory bladder responses as well as a generalized suppression of the defecation reflex. These fastigial effects were not at all dependent on the integrity of sympathetic pathways and consequently this particular cerebellar area is by no means only engaged in sympathetic control but rather seems to be involved in all kinds of autonomic mechanisms sympathetic as well as parasympathetic and also affecting combined autonomic-somatic responses like the defecation reflex.

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## Airway Effects of Slow Reacting Substance, Prostaglandin $F_2$ and Histamine in the Guinea-Pig

By

KJELL STRANDBERG and PER HEDQVIST

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### Abstract

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SRS,  $PGF_2$  and histamine were administered intravenously or as aerosols to artificially ventilated guinea pigs in order to assess their capacity to affect tracheal insufflation pressure measured by means of Konzett-Rossler technique. Independently of route of administration all three compounds increased tracheal insufflation pressure, SRS being the most potent one. Bilateral cervical vagotomy did not alter the effect. Relative to histamine SRS and  $PGF_{2\alpha}$  were considerably more active by aerosol administration than by intravenous injection. The aerosols had little or no effect on systemic blood pressure. On intravenous injection histamine decreased and SRS and  $PGF_2$  increased arterial blood pressure in a dose-dependent fashion. The airway effects of histamine were correlated to those on blood pressure whereas with SRS and  $PGF_{2\alpha}$  this was not seen when the blood pressure effects were marked. Preadministration of adrenaline or isoprenaline as aerosols antagonized the increase in insufflation pressure but not the effects on blood pressure produced by intravenously injected histamine or  $PGF_{2\alpha}$ .

It is concluded that SRS,  $PGF_2$  and histamine on intravenous or aerosol administration increase tracheal insufflation pressure in the guinea pig mainly by an action on airway tone. The data emphasize that SRS is a potent bronchoconstricting agent possibly of pathophysiological significance in guinea pig anaphylaxis.

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The appearance of a non-histamine smooth muscle stimulating principle, slow reacting substance (SRS), on anaphylactic reaction in the guinea pig lung was first observed by Kellaway and Trethewie (1940). Subsequently SRS has been demonstrated to be formed on histamine release in a variety of tissues and species including man (Chakravarty *et al* 1959, Chakravarty and Uvnäs 1960, Brocklehurst 1960). The mechanism of its formation seems to be closely related to the histamine release process (Chakravarty and Uvnäs 1960, Ånggård *et al* 1963, Strandberg 1971a).

The chemical identity of SRS is as yet unknown. However, SRS resembles the prostaglandins (PGs) in being a releasable, lipid-soluble, unsaturated hydroxy acid but it is not inactivated by prostaglandin dehydrogenase (Strandberg and Uvnäs 1971). The PGs together with histamine and SRS have recently been identified in perfusates from lung tissue undergoing anaphylaxis (Piper and Vane 1969, Piper and Walker 1973). Mainly due to their



constrictor action on bronchial smooth muscle SRS prostaglandin  $F_2$  ( $PGE_{22}$ ) and histamine have been suggested to serve as chemical mediators of anaphylactic bronchoconstriction (cf Collier and James 1970)

In the present investigation we have studied the action of SRS on airway tone in the anesthetized guinea pig and compared the effect with that of  $PGF_2$  and histamine. The compounds have been administered by intravenous injection or as aerosols and changes in the tracheal insufflation pressure have been recorded. Our data demonstrate that, independent of administration route SRS was the most effective compound in causing increased insufflation pressure. A preliminary account of part of the results has been presented elsewhere (Strandberg 1973)

### Methods and materials

Seventeen male albino guinea-pigs (650–935 g) were used for the study. They were anesthetized with sodium pentobarbitone (60 mg/kg) i.p. During the experiments supplements were given i.v. when needed. The trachea was cannulated and the animals were ventilated by the use of a Palmer constant volume respirator. The frequency was 40 strokes/min and the stroke volume (4–6 ml) was set according to the weight of the animals. Changes in the insufflation pressure at a constant airflow were measured with a Statham pressure transducer (P23 BC) connected to a side-arm of the tracheal cannula. When required, the expiratory arm of the tracheal cannula was clamped to facilitate the recovery by forced inflation. A catheter was inserted into the right internal jugular vein and heparin (500 IU/kg) was given. Systemic blood pressure was recorded from the right common carotid artery using a Statham pressure transducer (P23 AC). The recordings were made on a Grass model D polygraph. Aerosols were generated in a De Vilbiss nebulizer Model 35A nebulizing 0.5 ml/min of the fluid (5 ml). The nebulizer was connected to the air inlet channel of the ventilator. To minimize condensation of water all air passages were kept the shortest possible and were kept warm by lamps. The aerosol reached the tracheal cannula within 5 s.

SRS was obtained from cat paws perfused with a modified Tyrode solution containing compound 48/80 (1 µg/ml). It was purified by solvent extraction, silica acid and anion exchange chromatography as previously described (Strandberg and Lvnäs 1971). One unit of SRS has been defined as the threshold dose of SRS for a constrictor action on the isolated guinea-pig ileum (Strandberg 1971a). Prostaglandin  $F_2$  was a gift from Dr J Pike, Upjohn Co., Kalamazoo Michigan, U.S.A. Histamine chloride was used but the values given are in terms of the base.

### Results

#### *Intravenous administration*

On intravenous injection SRS,  $PGF_2$  and histamine produced an increase in tracheal insufflation pressure (Fig. 1). Usually the time lapse to reach the peak effect as well as the recovery period were shorter for histamine than for the other 2 compounds. Fig. 2 gives the dose-response curves for the effects on the insufflation pressure. Assuming that 1 unit of SRS is about 1 ng (Strandberg and Lvnäs 1971) the rank order of potency for the compounds was SRS > histamine >  $PGF_2$ . The doses of SRS and histamine causing 100% increase in insufflation pressure were significantly lower than that of  $PGF_2$  ( $p < 0.05$ ,  $n = 5$  according to Student's *t* test for paired variates).

SRS and  $PGF_{22}$  produced an initial drop in blood pressure succeeded by a prolonged and usually marked increase (Fig. 1). Histamine caused a more marked fall in blood pressure which was sometimes followed by a moderate increase. Histamine consistently produced dose-dependent changes of both tracheal insufflation pressure and systemic blood pressure. Usually this was also observed with SRS and  $PGF_2$ . However in some cases when the

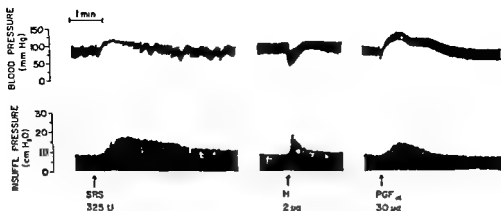


Fig. 1 Effects of i.v. injected SRS (1 unit = 1 ng),  $\text{PGF}_{2\alpha}$  and histamine on systemic blood pressure and tracheal insufflation pressure in a guinea pig (835 g) anesthetized with pentobarbitone

blood pressure effects were particularly marked and still dose related. SRS and  $\text{PGF}_{2\alpha}$  caused only small and variable changes in insufflation pressure.

The increase in insufflation pressure but not the effects on blood pressure produced by intravenously injected histamine or  $\text{PGF}_{2\alpha}$  was antagonized by a preceding 3 min administration of adrenaline or isoprenaline aerosols. Thus after adrenaline (25  $\mu\text{g}/\text{ml}$ ) the responses to histamine were 27–58 per cent ( $n=3$ ) and to  $\text{PGF}_{2\alpha}$  50–58 per cent ( $n=2$ ) of those recorded prior to administration of adrenaline. Corresponding figures for isoprenaline (25  $\mu\text{g}/\text{ml}$ ) ( $n=2$ ) were 10–30 per cent and 10–20 per cent respectively.

In an attempt to abolish the possible influence of reflex vagal activity on the action of the

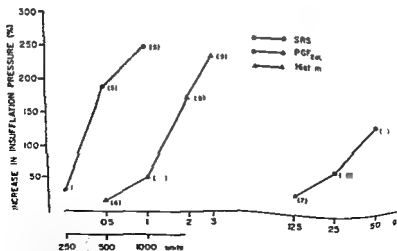


Fig. 2 Dose-response (peak effect) curves for the effects of intravenously administered SRS,  $\text{PGF}_{2\alpha}$  and histamine on the tracheal insufflation pressure. Mean values are given. Values in brackets are the number of observations.

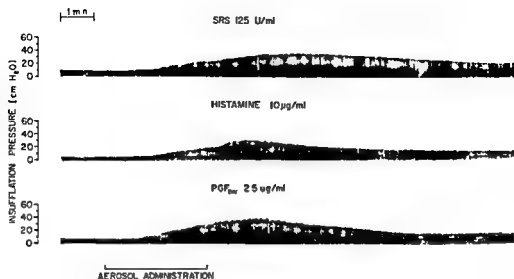


Fig 3 Effects of SRS, PGF<sub>2α</sub> and histamine as aerosols on the tracheal insufflation pressure in a guinea pig (760 g) anesthetized with pentobarbitone. Concentrations denote the respective concentrations in the aerosol generator.

compounds, bilateral cervical vagotomy was performed in 4 expts. However, this treatment did not significantly decrease either the basal insufflation pressure or the responses to the compounds.

#### Aerosol administration

Fig 3 illustrates the action of SRS, PGF<sub>2α</sub> and histamine administered as aerosols. They all produced a dose-dependent increase in tracheal insufflation pressure (Fig 4). PGF<sub>2α</sub> and histamine were largely equiactive but of considerable lower potency than SRS. Notably

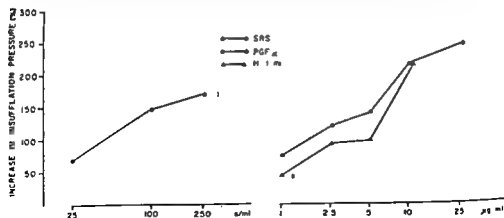


Fig 4 Dose response (peak effect) curves for the effects of SRS, PGF<sub>2α</sub> and histamine as aerosols on the tracheal insufflation pressure. Mean values are given. Within brackets are given the number of observations.

TABLE I Relative activities of SRS,  $\text{PGF}_{\alpha_2}$  and histamine on i.v. injection and on aerosol administration. The histamine dose and concentration respectively increasing tracheal insufflation pressure by 100 mm given as 1.0. The activities of SRS and  $\text{PGF}_{\alpha_2}$  were similarly calculated and are expressed in terms (ratios) of the histamine values. The data from Fig. 2 and 4 have been used for the calculations.

Compound	Intravenous injection	Aerosol administration
SRS	12	93
$\text{PGF}_{\alpha_2}$	0.1	2.5
Histamine	1.0	1.0

aerosol administration of the three compounds caused no effect or a slight decrease in systemic blood pressure.

In Table I the activities of SRS and  $\text{PGF}_{\alpha_2}$  in insufflation pressure are given relative to that of histamine. On this basis it seems that both SRS and  $\text{PGF}_{\alpha_2}$  were considerably more active via the aerosol route than given by i.v. injection. An overall comparison of the effects on insufflation pressure will however not be valid since the absolute quantities of the aerosols actually reaching the lower airways could not be determined.

### Discussion

The aim of this study was to analyze the actions on airway resistance of three potential anaphylactic mediator substances in the guinea pig, i.e. SRS,  $\text{PGF}_{\alpha_2}$  and histamine. For this purpose the substances were administered either by i.v. injection or as aerosols and changes in the tracheal insufflation pressure were taken to represent changes in airway resistance. It is true that this technique does not necessarily distinguish changes due to pulmonary congestion from those due to changes in bronchial tone (Widdicombe 1963). However, due to anatomical considerations, the particle size of the aerosols and the almost lack of systemic effects, it seems very likely that aerosol administration caused effects predominantly on the airway tone. The fact that the increase in insufflation pressure produced by i.v. injected histamine or  $\text{PGF}_{\alpha_2}$  was antagonized by aerosols of adrenaline or isoprenaline indicates that also the data obtained on intravenous injection at least in part reflect actions on the airway tone. In this context it should be noted that both sympathomimetic compounds relax bronchial muscle but differ conspicuously in their effects on the pulmonary and systemic circulation (cf. Aviado 1960). In addition, there was no true correlation between circulatory effects and effects on insufflation pressure after i.v. injections of SRS or  $\text{PGF}_{\alpha_2}$ . In fact, a marked blood pressure increasing effect was commonly associated with small and variable effects on tracheal insufflation pressure. Thus, there is no compelling reason to assume that circulatory events resulting from i.v. injection of SRS or  $\text{PGF}_{\alpha_2}$  are of any major importance in the production of increased insufflation pressure.

Irrespective of administration route, SRS was the most potent of the compounds studied. Like  $\text{PGF}_{\alpha_2}$ , the effect of SRS on the tracheal insufflation pressure was more pronounced on aerosol administration than on i.v. injection when the results were related to the effects of histamine. Similar data have been reported for the bronchodilator action of  $\text{PGF}_{\alpha_2}$  in com-

parison to the effect of isoprenaline (Large Leswell and Maxwell 1969) The lung has a high capacity to inactivate PGs independently of whether they are administered directly to lung tissue or given as i.v. injection (Ånggård and Samuelsson 1964 Ferreira and Vane 1967) Concerning SRS both guinea pig serum and lung tissue have been shown to inactivate SRS (Strandberg 1971 b) The relatively higher potency of SRS and  $\text{PGF}_{2\alpha}$  as aerosols compared to i.v. injection is therefore probably due to differences in diffusion distance to the target area

On intravenous injection SRS and  $\text{PGF}_{2\alpha}$  produced an increase in the systemic blood pressure A similar effect of  $\text{PGF}_{2\alpha}$  is seen in the dog whereas  $\text{PGF}_{2\alpha}$  has a depressor action in the cat and rabbit (cf Karim and Somers 1972) In the dog the pressor effect has been attributed to peripheral venoconstriction leading to an increased venous return and thereby an increase in the cardiac output (Du Charme Weeks and Montgomery 1968) However Nakano and Cole (1969) considered the increased cardiac output a result of the increased myocardial contractility caused by  $\text{PGF}_{2\alpha}$  The depressor effect of  $\text{PGF}_{2\alpha}$  and of the other compounds occasionally observed on aerosol administration in the present study may be a circulatory consequence of the prevailing bronchoconstriction

Anaphylactic bronchoconstriction in the guinea pig is considered to be mediated by the release and formation of bronchoconstricting substances i.e. principally histamine SRS  $\text{PGF}_{2\alpha}$  and kinins in the lung (cf Collier and James 1970) Using a variety of chemical antagonists Collier and James (1967) tried to estimate the relative contribution of some of the possible mediators in the anaphylactic reaction They confirmed the generally accepted view that antihistamines render protection and could also show that some non steroidal anti inflammatory compounds e.g. aspirin and meclofenamate gave additional protection The latter findings were taken to indicate that SRS and kinins contribute to the anaphylactic bronchoconstriction since it was shown that the anti inflammatory compounds used antagonized the actions of partially purified SRS and bradykinin administered i.v. In the light of recent observations that aspirin, indomethacin, meclofenamate and allied substances are potent inhibitors of prostaglandin synthetase (Vane 1971 1973) the results could equally well suggest inhibition of the production of PGs during anaphylaxis as also pointed out by Collier (1971) In fact Collier (1971) proposed that SRS and kinins exert their effects in the guinea pig lung via activation of PG biosynthesis However peroral pretreatment of sensitized guinea pig with high doses of indomethacin does not protect the animals from developing respiratory distress and convulsions on inhalation of antigen although the production of  $\text{PGF}_{2\alpha}$  is inhibited (Strandberg and Hamberg 1974) This seems to indicate that at least  $\text{PGF}_{2\alpha}$  is no major mediator in anaphylactic bronchoconstriction in the guinea pig It is even questionable if histamine is of prime importance for this reaction since it has been demonstrated that the protective effect of the anti histaminic compound mepyramine is abolished by adrenalectomy and partially inhibited by  $\beta$  adrenoceptor blockade (Bernauer Hahn and Gieritz 1969 Bernauer Gossow and Hahn 1970) The present data emphasize that SRS is a potent bronchoconstrictor substance in the guinea pig Taken together the data suggest that SRS may be of pathophysiological significance in guinea pig anaphylaxis

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## Postnatal Ontogenetic Development of Neurogenic and Myogenic Control in the Rat Portal Vein

By

BENGT LJUNG and DOROTHY STAGE (McMURPHY)

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### Abstract

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The postnatal ontogenetic development of neuro effector control in vascular smooth muscle of the single unit type has been studied in the rat portal vein. Contractile activity was recorded isometrically in isolated preparations from rats 2-38 days of age and in adults rats. Spontaneous activity characteristic of the adult portal vein appeared abruptly during the third postnatal week. Whereas induced responses to noradrenaline (NA) and acetylcholine (Ach) appeared early during the first week and responses to transmural nerve stimulation occurred at the end of the first week. The appearance of spontaneous activity was accompanied by significant increases in sensitivity to NA ( $\log ED_{50}$ ) and to transmural nerve stimulation (frequency giving half maximum response) but not to Ach. Also maximum response for NA and nerve stimulation relative to Ach responses tended to increase during the first three weeks. It is concluded that the development of a mechanism supporting myogenic propagation as revealed by highly synchronized spontaneous contractions is an important factor for promoting the effectiveness of sympathico-adrenergic control in this type of vascular smooth muscle.

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In recent years the neuro effector function of the isolated portal vein has been investigated in some detail since its properties apparently make it a suitable model for the study of neurogenic control of propagating vascular smooth muscle (see Ljung 1970, Johansson *et al* 1972). The longitudinal smooth muscle in the portal vein of the adult rat exhibits synchronized spontaneous contractions of myogenic origin and propagation (Johansson and Ljung 1967). Such myogenic cell to cell propagation contributes to the effectiveness of the sympathico-adrenergic control of this type of smooth muscle by spreading induced activity from muscle cells in the vicinity of the terminal nerve plexus to other non-innervated cells located further out in the longitudinal layer. This spread of activity is important since noradrenaline (NA) whether released from the terminal nerve plexus or exogenously administered seems to primarily activate the innervated muscle cells in the portal vein (Johansson and Ljung 1968, Johansson *et al* 1970, Ljung 1970, Johansson *et al* 1972, Ljung, Bevan and Su 1973).

The media of the portal vein from the adult rat is divided into a thin inner circular layer and a thicker outer longitudinal layer. The functionally important terminal vasomotor nerve supply is arranged in a two-dimensional adrenergic plexus between the muscle layers and does not ramify within the muscle tissue (Johansson *et al* 1970 Ljung *et al* 1973). However at birth the double layered smooth muscle configuration of the adult rat portal vein is not found, but develops during the first three postnatal weeks. At 10 days of age the splitting of the media into two distinct and separate muscle zones, the inner circular and the outer longitudinal is clearly distinguishable. Unmyelinated nerve fibers are present in the adventitia at birth, but have not been found between the two medial layers of the developing vein when studied in rats up to the 6th week of life (Ts'ao, Glagov and Kelsey 1971).

In the light of these histological observations on the developing rat portal vein it appeared to us that the vessel offers the possibility of studying the ontogenesis of neuro-effector function in propagating vascular smooth muscle. In the present study the appearance and development of spontaneous activity and induced responses are analyzed and compared in isolated portal vein preparations from young rats. The results show dramatic changes in the responses of this vascular tissue during the first few weeks of postnatal life.

### Methods

Portal vein preparations from 331 young rats (2-38 days of age) and 15 mother rats of the Sprague-Dawley strain were studied in these experiments. Division of the rats by age, weight and length is noted in Table I. The animals were killed either by decapitation or by a blow to the neck. The portal vein was dissected under a dissection microscope tied at both ends and mounted under longitudinal tension in an organ bath containing 40 ml of Krebs solution (composition see below). Initial resting force exerted on the smooth muscle was adjusted to that length at which optimal active force had been found to be developed in preliminary experiments. This preload varied with age between 0.5-4 mN (week 1 and adults, respectively). The average increase in applied passive force was approximately 0.4 mN per week in the young rats. The muscle was allowed to stabilize for 60 min and the preload was adjusted during this accommodation period. Isometric longitudinal contractile activity was recorded on a Grass polygraph (model 7) by means of a Grass force-displacement transducer (FT 03C) and the force signal was electronically integrated on a separate channel which continuously provided values for mean contractile force. Induced responses were elicited at the mean force developed during the period of stimulation minus the mean force during the preceding 3 min control period.

The adrenergic nerve plexus of the portal vein preparation was activated by transmural field stimulation (NS) during 30 s periods by use of a Grass Stimulator model 54B which provided square wave impulses of 0.8 ms duration and 15 V in amplitude between platinum electrodes on either side of the preparation. In control experiments responses to these stimulation parameters were found to be blocked by tetrodotoxin or adrenergic blocking agents. The response of the preparations to different impulse rates ranging from 0.25 to 100 Hz was determined separately for each frequency by starting at the lowest setting and increasing progressively until a maximum response was elicited. The interval between each stimulation was at least 10 min to allow time for complete recovery. These responses determined for each frequency were then expressed as a percentage of (1) maximum contractile response elicited by norepinephrine (NA) in that particular preparation and (2) the maximum NS response set at 100 per cent. The preparations were divided into groups according to age in weeks and mean values  $\pm$  SE were calculated for each frequency within the various groups. In this way frequency response curves were obtained and plotted at different ages.

Dose response curves for exogenous administered NA and acetylcholine (ACh) were determined in the following way. The respective drug in appropriate concentration was added directly to the bath for a 3 min exposure period. The drug was then washed out by repeated rinses and a 10-15 min interval was



TABLE 1 Division of rats by age

	Age groups (weeks)						A
	1	2	3	4	5	6	
Age (days)	2-7	8-14	15-21	22-28	29-35	36-38	Adults
Weight (g $\pm$ S.E.)	10 $\pm$ 1	24 $\pm$ 2	38 $\pm$ 2	61 $\pm$ 2	96 $\pm$ 3	129 $\pm$ 4	306 $\pm$ 6
Crown-rump length (mm $\pm$ S.E.)	63 $\pm$ 2	86 $\pm$ 2	106 $\pm$ 2	148 $\pm$ 2	150 $\pm$ 2	167 $\pm$ 3	234 $\pm$ 7
Number	27	30	26	19	18	11	15

allowed for complete return of activity to the baseline level before a 10 times higher drug concentration was given. In this way a range of individual responses to drugs administered was determined in concentrations from below threshold up to supramaximum concentrations. The experimental values were expressed as a per cent of the maximum response to the respective agent for each preparation. Dose response curves for the different age groups were obtained by plotting the response values (mean  $\pm$  S.E.) against concentration. In addition a calculator program was used to determine the least squares fit of the values from each expt to a true hyperbolic function.  $ED_{50}$  values were determined from these dose response functions as that concentration at which a 50 per cent response would be obtained and expressed in logarithmic form ( $\log ED_{50}$ ).

Maximum peak contractile force for spontaneous activity as measured at the end of the initial accommodation period and for responses to nerve stimulation and exogenous NA and Ach was determined and expressed in absolute values as well as in per cent of that maximum NA response which was elicited in all expts.

At the end of each expt the length of the stretched preparation was measured and the wet weight was determined on a Cahn electrobalance after the external fluid had been removed by wiping the tissue against a glass surface until no moist trace was left behind. The cross sectional area ( $mm^2$ ) and maximum tension ( $mN/mm$ ) were calculated assuming a density of 1.0.

Comparison of the mean values between the various age groups was made by use of the Student's *t* test. A statistically significant difference was considered to exist between the compared groups when the *p* value was less than 0.05. The age of onset (mean  $\pm$  S.E.) for a particular response was obtained by sequentially determining the ratio between the number of responding tissues and the total number of tissues each day of life. This ratio was considered as a cumulative probability distribution function of time occurring during the transition period from complete unresponsiveness to the day that all tissues responded.

The Krebs solution had the following composition in mM: NaCl 120, KCl 4.73,  $NaHCO_3$  15.5,  $KH_2PO_4$  1.19, MgCl<sub>2</sub> 1.19,  $CaCl_2$  2.49, Glucose 11.5 and  $CaNa_2$ -versenate 0.026. It was continuously bubbled with 4 per cent  $CO_2$  in  $O_2$  to maintain a pH of 7.35 at the thermostatically set temperature of 38°C.

The drugs used were noradrenaline bitartrate (nor Exadrin conc Astra AB Södertälje Sweden), acetylcholine chloride (Merck West Germany) and tetrodotoxin (Sigma Chemical Co. St. Louis Mo). Freshly prepared saline diluted drugs in a volume of 0.4 ml were injected directly into the 40 ml bath to give the desired concentration.

## Results

*Onset of portal vein responses in neonatal rats* The histograms in Fig. 1 illustrate the age of onset for spontaneous activity and responsiveness to exogenously administered noradrenaline (NA), acetylcholine (Ach) and to transmural field stimulation of the nerve supply (NS) in the isolated portal vein of the neonatal rat. No spontaneous activity was found during the first 2 weeks of postnatal life. In a transition period between the 15th and 18th days of age spontaneous activity appeared in an increasing number of vessels so that after the 19th day it was evident in all preparations. The average age of onset was  $16.5 \pm 1.6$  (days  $\pm$  S.E.). Portal vein responses induced by exogenous NA as well as by Ach occurred much earlier at

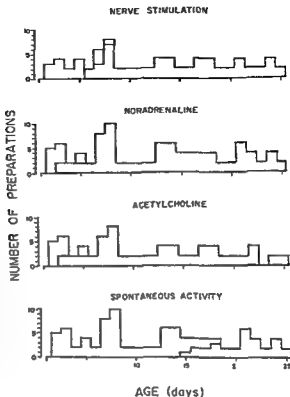


Fig. 1 Age of rats at onset of portal vein responses. Number of preparations exhibiting spontaneous activity and responding to exogenous acetylcholine, exogenous noradrenaline and 10 transmurial nerve stimulation (filled in squares) and number of those not responding (open squares) plotted against age (days). Note early appearance of induced portal vein responses in comparison with later onset of spontaneous activity.

an average age of  $3.7 \pm 1.3$  (days  $\pm$  S.E.). Transmurial field stimulation induced neurogenic responses of the portal vein from the 6th day on with an average age of onset of  $6.1 \pm 1.3$  (days  $\pm$  S.E.). Thus portal vein responsiveness to NA, Ach and nerve stimulation was not a consistent finding during the first week of postnatal life, but after the 8th day of age all portal vein preparations responded to all three stimuli.

The development of spontaneous activity and induced responses to NA, Ach and NS in the portal vein of maturing rats is described in the following sections with regard to maximum force and sensitivity to the stimulating influences.

**Development of spontaneous activity.** As illustrated in Fig. 1, no spontaneous activity was recorded in the isolated portal vein of rats during the first 2 weeks of postnatal life. Between 15 and 18 days of age spontaneous phasic activity first appeared and was characterized by erratic contractions of variable amplitude occurring at irregular intervals (Fig. 2 left diagram). During this transition period 8 out of the 16 preparations exhibited this type of spontaneous myogenic activity as recorded at the end of the initial 1 h accommodation period. In an additional 4 out of these 16 preparations phasic activity appeared at the end of the NA and/or Ach experiments, even though the drug was apparently removed by repeated rinses. Phasic contractile activity of low frequency occurred in a few preparations from younger rats (13 to 15 days) that were allowed to stand overnight in a bath of normal Krebs solution. After 19 days of age all portal vein preparations exhibited spontaneous

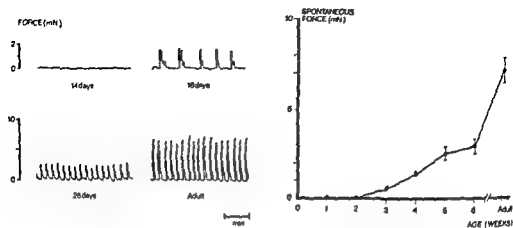


Fig 2 Development of spontaneous activity in rat portal vein. Recordings illustrating pattern of spontaneous activity at different stages of development (left diagram). Spontaneous force (mean  $\pm$  S.E.) plotted against age in weeks (right diagram). For number of observations see Table 1. Note gradual increase in mean force with advancing age.

myogenic activity. Gradually a more consistent pattern of regular phasic synchronized activity was established so that by the third to fourth week regular phasic contractions of comparable magnitude and at a rate of approximately 2–4 per min occurred throughout the entire experiment (Fig. 2 left diagram).

The mean force exerted by spontaneous contractile activity as measured at the end of the initial accommodation period progressively increased with age from zero during the first two weeks up to nearly 3 mN by the end of the sixth week. This latter value was about half of the spontaneous force exerted by the adult portal vein (Fig. 2 right diagram). The peak force of spontaneous activity and of induced responses was expressed as a percentage of the maximum force developed in response to NA in each experiment so that comparison could be made between the magnitudes of maximum responses to different exogenous stimuli at different ages. This percentage of spontaneous activity increased from zero during the first two weeks up to 1/3 of maximum NA response during the 4th to 6th weeks and was half of the maximum NA response in adult rats (see Fig. 8). Thus the basal level of spontaneous activity as expressed in terms of maximum contractile force increased with age in the developing rat portal vein.

The synchronized spontaneous contractions were not affected by complete blockade of the  $\alpha$  adrenergic receptors or by neuronal blockade with tetrodotoxin. In Fig. 3 at the left spontaneous activity and a response to transmural nerve stimulation (4 Hz) are illustrated before tetrodotoxin block. After a 30 min exposure to tetrodotoxin ( $10^{-6}$  M) the frequency and amplitude of the spontaneous contractions were unchanged whereas the nerve response was completely blocked. Sixty min after the drug had been washed out the spontaneous activity remained the same and the nerve response returned to its previous level.

In the following sections it will be seen that the onset of spontaneous activity after 2 weeks of age coincided with a marked increase in sensitivity to exogenous NA and to vaso-

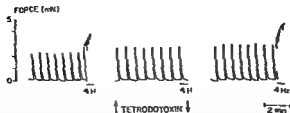


Fig 3 Recordings of spontaneous activity and responses to transmural stimulation (4 Hz 0.8 ms and 15 V) in a 2 day old rat portal vein preparation before (left panel) 30 min after (middle panel) the administration of tetrodotoxin ( $10^{-4}$  M) and 1 h after wash out of the drug (right panel). Note that the response to transmural stimulation is blocked by tetrodotoxin while spontaneous activity is unaffected.

**motor nerve stimulation** This age apparently represented a milestone in the maturation of the rat portal vein.

**Portal vein response to noradrenaline** Actual tracings from experiments on the rat portal vein before and after the appearance of spontaneous activity (8 and 21 days of age respectively) and on a preparation from a mother rat illustrate the contractile responses to exogenous NA (Fig 4). The response pattern in the young portal vein without spontaneous activity was characterized by a smooth increase in force from baseline up to a plateau level which persisted throughout the exposure period. The first response above threshold was often comparatively great in magnitude. Subsequent 10-fold increases in NA concentration often gave only small additional increases in amplitude. After the appearance of spontaneous activity (middle and lower recordings) the pattern of the contractile responses was altered. First at low NA concentrations the frequency of the phasic contractions increased. Then the

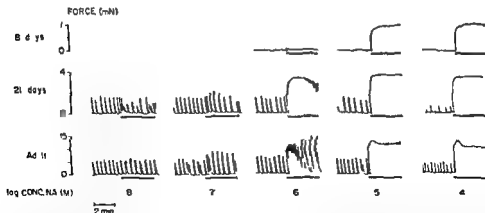


Fig 4 Recordings of myogenic activity and electory responses to noradrenaline (NA) in portal vein preparations from rats 8 and 21 days of age respectively (upper and middle panels) and an adult rat (lower panel). Agonist administered in progressively increasing concentrations ( $10^{-8}$  to  $10^{-4}$  M) during 3 min periods as indicated by bar length. Note change in NA sensitivity and in response pattern after appearance of spontaneous activity.

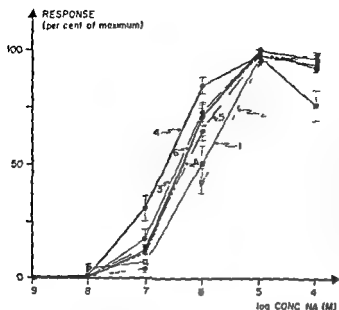


Fig 5 Relationships between nor adrenaline (NA) concentration and integrated force response expressed as per cent of maximum response (mean  $\pm$  S.E.) in portal vein preparations from young rats grouped according to age in weeks (1-6) and from adult rats (A). Number of observations in each group 1-14 2-18 3-16 4-15 5-12 6-7 A-9

amplitude of these contractions also increased above the basal level (NA conc  $10^{-8}$  M). The next stage was one of incomplete tetanus characterized by the rapid development of a smooth increase in force which gave way to phasic contractions of augmented amplitude. The final stage resembled a complete tetanus marked by the rapid development of force which plateaued at a stable level throughout the exposure period. This last tetanic stage occurred at a NA concentration of  $10^{-6}$  M. Thus the progression of development of responses in a concentration dependent manner appeared more gradual in those preparations with spontaneous activity.

All responses to NA were quantitated as the increase in mean active force (see Methods) and expressed as a percentage of the maximum NA response. Dose response relationships for NA ( $10^{-10}$  to  $10^{-6}$  M) were determined in portal vein preparations from 88 young rats (2-38 days of age) and in 9 adult rats. These 98 tissues were then grouped according to age in weeks (Table I) and mean integrated response values  $\pm$  S.E. were determined for each NA concentration (Fig 5). Except for week 4 which was furthest to the left hardly any variation was found between the dose response curves after 3 weeks of age. Those of the first two weeks were displaced to the right. Thus in order to elicit a given response a higher NA concentration was required in these youngest rats. Furthermore it was seen from Fig 5 that maximum responses were obtained at a NA concentration of  $10^{-6}$  M.

In an additional 48 expts where other means of stimulation were primarily employed NA in the concentration of  $10^{-6}$  M was also administered so that maximum active force in response to NA was measured in a total number of 146 preparations. This maximum force increased progressively with age from a mean value of 0.3 up to 9 mN (weeks 1 and 6 respectively) reaching a peak value of 13.5 mN in adult rats (Fig 6). The differences between these maximum force values were statistically significant for all age groups. The cross sectional area of each preparation was calculated from its measured length and weight

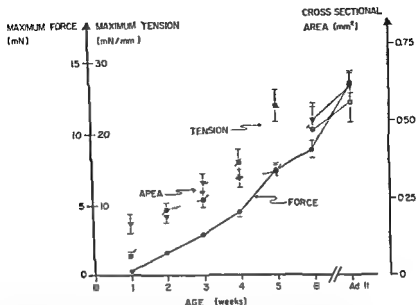


Fig 6 Maximum force response to exogenous noradrenaline (●—●) cross sectional area (▼—▼) and calculated maximum tension (□—□) in portal vein preparations from rats at different ages. Mean  $\pm$  SE indicated for each age group. For number of observations see Table I.

assuming a density of 1.0 (see Methods). Probably due to the technical difficulties involved in obtaining these measurements in small tissues, statistically significant differences for the mean values of cross sectional area were not detected between all age groups. However, the trend was that area increased with age from a mean value of  $0.17 \text{ mm}^2$  (week 1) up to  $0.62 \text{ mm}^2$  (adults) (Fig. 6). The values for maximum active force and cross sectional area, albeit crudely determined, allowed for the calculation of maximum tension. Again, although a definite trend of increase with age was seen from a mean value of  $3 \text{ mN/mm}^2$  (week 1) up to  $24 \text{ mN/mm}^2$  (adults), statistical differences between all age groups were not obtained.

The tissue sensitivity to NA was determined as the  $\text{ED}_{50}$  value in each dose response experiment (see Methods). Means of the  $\log \text{ED}_{50}$  values were calculated for each week (Fig. 7). A distinct increase in NA sensitivity occurred after the 2nd week, i.e. that concentration of NA required to produce a 50 per cent response decreased. All mean  $\log \text{ED}_{50}$  values from the 3rd week onto adults were significantly lower than those of the 1st and 2nd weeks. The mean  $\log \text{ED}_{50}$  value for the 4th week was lowest when compared with all other age groups. As mentioned above, the increase in  $\text{ED}_{50}$  for NA after the 2nd week occurred simultaneously with the appearance of spontaneous activity (Fig. 7).

**Portal vein response to acetylcholine** Exogenous Ach caused excitatory responses of the portal vein from the 3rd day on (Fig. 1). The different patterns of contractile activity induced by graded concentrations of Ach at the different stages of portal vein maturity resembled the NA responses illustrated in Fig. 4. The progression of Ach induced responses with increasing Ach concentration was more gradual than for NA.

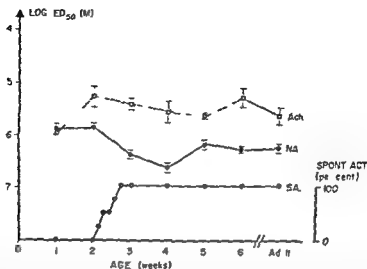


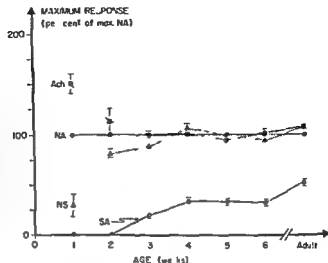
Fig. 7 Log  $ED_{50}$  values (mean  $\pm$  S.E.) obtained from a least squares fit of integrated response values for noradrenaline (NA) and acetylcholine (Ach) to true hyperbolic function in developing rat portal vein (upper curves). Lower curves represent percentage of all preparations displaying spontaneous activity (S.A.) at different ages. Number of observations for NA see Fig. 5. Ach week 1-4 2-6 3-10 4-4 5-4 6-4 adult-4 and S.A. see Table 1. Note the significant decrease in log  $ED_{50}$  for NA but not for Ach, for all age groups after week 2 when spontaneous activity appeared.

Dose response relationships for exogenously administered Ach in concentrations ranging from  $10^{-8}$  to  $10^{-5}$  M were determined in 38 young rats (2-37 days of age) and in 4 adult rats. Tissue sensitivity to Ach was determined as the  $ED_{50}$  value in each dose response experiment (see Methods). Mean log  $ED_{50}$  values were calculated and grouped according to age in weeks (Fig. 7). In comparing Ach with NA the portal vein was less sensitive to Ach in that significantly higher Ach concentrations were required to produce a 50 per cent response in all age groups except week 1. From the second week on, the sensitivity of the young portal vein to Ach did not change with advancing age.

The mean values for maximum Ach response increased progressively from 0.3 up to 9.7 mN (weeks 1 and 6 respectively) reaching the value of 15.9 mN in adult rats. The differences between these increases were statistically significant for all age groups. Mean values of maximum force for Ach expressed as a percentage of the maximum response to NA  $10^{-5}$  M in each experiment are illustrated in Fig. 8. The maximum Ach response was significantly greater than that for NA during the first two weeks but very close to the NA value from the third week on.

*Portal vein response to nerve stimulation.* Actual recordings from transmural stimulation experiments on isolated portal veins from rats of different ages are illustrated in Fig. 9. In the youngest rats responses were obtained only at high impulse rates and after a considerable delay sometimes lasting longer than the 30 s stimulation period. The pattern of contractile response in these preparations without spontaneous activity resembled the NA response in that there was a smooth increase in force from baseline up to a peak level. Following stimulation at high frequencies there was often a biphasic decrease in force (Fig. 9 top recordings). When the spontaneous activity appeared after the second week

Fig 8 Maximum force (mean  $\pm$  S.E.) responses for spontaneous activity (SA) exogenous noradrenaline (NA) acetylcholine (Ach) and transmural nerve stimulation (NS) expressed as a percentage of max NA in portal veins from rats at different ages. Number of preparations for SA see Table I. Ach see Fig 7. NS see Fig. 10.



neurogenic responses were obtained at much lower stimulation frequencies and occurred nearly instantaneously with the stimulation. At low and moderate nerve impulse frequencies the phasic contractions increased in rate and amplitude. At high frequencies they tended to fuse giving a tetanus-like response (Fig 9 middle and lower recordings). Maximum force occurred at an impulse frequency of 32 Hz in both young and adult rats.

Curves relating neurogenic response expressed as a percentage of the maximum NA response to stimulation frequency were determined in 101 young rats (2–38 days of age) and in 11 adult rats. The mean response values  $\pm$  S.E. were plotted according to age in weeks (Fig 10 left diagram). During the first two weeks the responses as related to maximum NA response were very small and elicited only after exceedingly high non-physiologic impulse rates. Thereafter the threshold frequency was markedly lowered and from the third week on the relative amplitude of neural responses was higher without obvious variation between the different age groups. Maximum responses were elicited by a frequency of 32 Hz in all age groups.

As for NA and Ach the amplitude of the maximum NS response increased progressively with age from a mean value of 0.1 up to 8 mN (weeks 1 and 3 respectively) reaching a peak value of 14 mN in adult rats. The differences between these mean values were statistically significant for all age groups except between weeks 5 and 3. Mean values of the maximum neurogenic force expressed as a percentage of the maximum NA response in each experiment are shown in Fig 8. The mean maximum NS response was significantly less than that for NA during the first 3 weeks but not different from NA and Ach values from the 4th week on.

The frequency response curves of Fig 10 left diagram indicate that not only the maximum amplitude but also the sensitivity to change in nerve impulse frequency varied with age. In Fig 10 right diagram this is more clearly illustrated when the neurogenic responses are expressed as a percentage of maximum NS responses and related to stimulation frequency on a linear scale. The curves for the 1st and 2nd weeks were clearly displaced to the right.



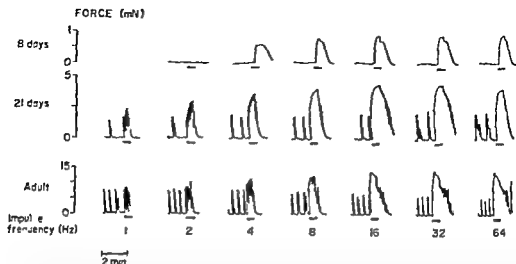


Fig. 9 Tracings of responses to transmural nerve stimulation at graded frequencies (values at bottom) (0.8 ms, 15 V) in portal vein preparations from rats 8 and 21 days of age respectively (upper and middle panels) and from an adult rat (lower panel). Stimulation applied for 30 s intervals indicated by bar length. Note changes in low frequency sensitivity in response pattern and in delay time for onset of response after appearance of spontaneous activity.

of the curves for older age groups which in turn showed little variation. Thus a higher frequency was needed to elicit a given relative response in the youngest rats. The frequency required to give a 50 per cent response can be estimated by inspection of the curves in Fig. 10 right diagram. This value was 13 and 7 Hz for weeks 1 and 2 respectively and

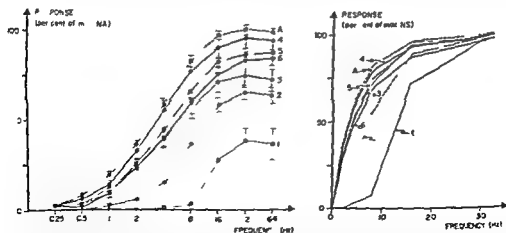


Fig. 10 Relationship between nerve stimulation frequency and integrated contractile response expressed as a percentage (mean  $\pm$  S.E.) of maximum response to exogenous noradrenaline (max NA) in left diagram and expressed as a percentage of maximum neurogenic response (max NS) at right. Portal vein preparations from young rats grouped according to age in weeks (1-6) and from adult rats (A). Number of observations in each group: 1-5 = 23, 3-20, 4-15, 5-14, 6-7, A-11. Note that sensitivity to nerve stimulation increases during the first 3 weeks.

ranged between 3.2 to 4.6 Hz for the older age groups. This indicates a definite increase in sensitivity to NS after the 2nd week which appeared coincidentally with the onset of spontaneous activity and the increase in NA sensitivity (see Fig. 7).

### Discussion

The longitudinal muscle layer of the portal vein from various adult laboratory animals exhibits spontaneous phasic activity when studied *in vitro*. The synchronization of the electrical activity within the tissue which is required for effective coordinated contractions has been demonstrated in the adult rat portal vein to be due to myogenic cell-to-cell propagation (Johansson and Ljung 1967; Ljung and Stage 1970).

In the isolated postnatal rat portal vein passively stretched according to age to give optimal active force, phasic activity first appeared during the third week of life. These spontaneous contractions were not affected by exposure to tetrodotoxin in concentrations which eliminated nerve responses (Fig. 3) and thus they are in all probability dependent on myogenic mechanisms. The apparent lack of single unit behavior during the first two postnatal weeks probably reflects the absence of effective myogenic spread of excitation in the very young portal vein. Developing smooth muscle cells grown in tissue culture have been found to form aggregates which begin to contract in synchrony as the cells become electrotonically interconnected (Purves, Mark and Burnstock 1973). Likewise it seems reasonable to surmise that the appearance of synchronized activity in the developing smooth muscle of the rat portal vein at around the 15th day might reflect the establishment of functioning bundles of coupled muscle cells.

However, in addition to myogenic spread of excitation, coordinated phasic contractions require activity that is rhythmically induced from groups of muscle cells with pacemaker function. Such activity can be evoked and/or accelerated in the adult portal vein by depolarizing influences such as exogenous NA (Ljung and Stage 1970) or stretch (Johansson and Mellander 1975). In the present experiments coordinated phasic contractile activity was sometimes induced at an age of 15–18 days but never during the first two weeks of life regardless of the degree of stretch.

It is of great interest that in the third week of life coincidental to the appearance of spontaneous activity, the sensitivities to exogenous NA and to transmural nerve stimulation were found to increase significantly. In a series of previous studies it has been shown that the ability of the smooth muscle of the adult rat portal vein to support propagation contributes in an important manner to the effectiveness of the sympathico-adrenergic control of the vessel. During nerve activity the released transmitter substance NA apparently reaches only smooth muscle cells near the terminal adrenergic nerve plexus in the high peak concentrations which determine the neurogenic responses in the adult rat portal vein (Ljung 1969, 1970; Johansson *et al.* 1972). In addition, the functionally important  $\alpha$  receptors seem to be mainly restricted to these comparatively few innervated cells (Johansson *et al.* 1970; Ljung *et al.* 1973; Bevan and Ljung 1973). Therefore, myogenic propagation of locally induced excitation is needed for the recruitment of the succeeding non-innervated

smooth muscle cells to respond effectively not only to nerve stimulation but also to exogenous NA. As a consequence the increased sensitivity to adrenergic stimulation found after the second week can probably be attributed to the development of functionally important myogenic propagation mechanisms in the maturing vascular muscle.

It appears that such an increase in functionally relevant spread of activity within vascular smooth muscle does not necessarily parallel the relative abundance of intercellular connections or the passive electrical properties of the muscle tissue. In a series of studies (Mekata 1971, 1974 and Mekata and Niu 1972) it has been shown that the smooth muscle of elastic arteries has cable properties and length constants of considerable magnitude. However, action potentials cannot be triggered (see Mekata 1974) and it seems that the lack of such an efficient regenerating mechanism may account for the apparent absence of longitudinal spread of induced activity in large arteries. This is in contrast to the situation in the adult portal vein and peripheral arteries where functional spread of induced activity has been demonstrated (Bevan and Ljung 1974). Thus it seems possible that the occurrence of spontaneous activity and the concomitant increase in sensitivity to  $\alpha$  receptor stimulation might reflect a change in membrane excitability and/or the establishment of muscle cell interconnections in the developing portal vein.

In contrast to adrenergic stimulation the sensitivity to exogenous Ach was not found to increase during development. The results of a recent study indicated that, as opposed to the sensitivity to  $\alpha$  adrenergic stimulation, the sensitivity to non adrenergic influences such as Ach and  $K_+$  is evenly distributed within the smooth muscle (Bevan and Ljung 1973). Thus it may be concluded that myogenic propagation in this type of vascular smooth muscle is less important in promoting the effectiveness of cholinergic than of adrenergic responses.

The greater sensitivity of the maturing smooth muscle to  $\alpha$  adrenergic stimulation might course be due to alterations in the properties of  $\alpha$  receptors or increased reactivity of the  $\alpha$  processes linking the agonist receptor interaction to the contractile response. However, previous studies of the ontogenetic development of both  $\alpha$  and  $\beta$  adrenergic and cholinergic receptor function in isolated tissues without spontaneous activity have shown that the tissue sensitivity to the agonists NA and Ach did not change with maturation (McMurphy and Boreus 1968, Boreus and McMurphy 1971, Boreus, Malmfors, McMurphy and Olson unpubl. results). This further supports our thesis that it is the appearance of efficient myogenic propagation in the developing portal vein which is responsible for the increased sensitivity to NA.

The maturation of the smooth muscle of the portal vein was not only reflected by the development of spontaneous activity and by the alteration in sensitivity to exogenous NA but also by a progressive increase of maximum force per unit cross sectional area exerted spontaneously and in response to exogenous NA and Ach with advancing age (cf. Fig. 2, 6 and 8). The latter was not dependent upon myogenic propagation but seemed to reveal a structural or functional alteration in the smooth muscle itself. It may be that the ratio between smooth muscle content and the total cross sectional area of the vessel increases during the entire period studied or that each unit of smooth muscle becomes more efficient in generating force. At present sufficient morphological information is not available to settle this question. However, the neonatal rat portal vein has been studied with electron

microscopy (Ts Ao *et al* 1971) It was found that the adult pattern of a double layered muscular structure with division into inner circular and outer longitudinal smooth muscle layers was not present at birth but developed during the first 3 postnatal weeks Partitioning of the media was found to start at 3 days of age A clear orientation of the two muscle zones separated by an intervening fibrous layer was established by 7-10 days and was further differentiated in the ensuing two weeks

A remarkably similar time course for the development of skeletal muscle of the neonatal rat hindlimb into two functional types fast and slow muscle has been observed to occur during the first 3 weeks of postnatal life An increase in sensitivity of vascular smooth muscle to NA occurred simultaneously so that by the time of weaning at about 21 days the reactivity to exogenously administered NA was almost the same as in the adult rat (Gray 1972)

Responses to nerve stimulation at high frequencies first appeared towards the end of the first week of life These responses which could be blocked pharmacologically (see Fig 3) demonstrated the presence of functioning adrenergic nerves at this age The sensitivity to transmural nerve stimulation increased considerably during the first two weeks as apparent from the shift of the frequency response curves to the left (Fig 10 right diagram) and by the increased response amplitude relative to the maximum NA response (Fig 10 left diagram and Fig 8) In addition the considerable delay existing between onset of nerve stimulation and response was greatly shortened after the second week (see Fig 9) The outgrowth of nerves appears to be particularly important for the observed increases in neural responses between the first and the second week In this period of time the maximum response to nerve stimulation expressed as a percentage of max NA was almost doubled and the frequency giving 50 per cent of the maximum NS response was approximately halved After the second week the development of myogenic propagation must be an important factor for the enhancement of the neurogenic responses In fact the importance of this mechanism in the case of nerve induced activity must be at least as great as it is for exogenous NA During physiological nerve impulse rates the released transmitter will apparently reach only the nearest muscle cells in effective concentrations (Johansson *et al* 1972) Therefore myogenic propagation is required for normal activation of the non innervated muscle cells

In the thin wall of the young portal vein without spontaneous activity the released transmitter may accumulate during prolonged stimulation at high frequencies so that weak responses were elicited after long delays After the appearance of spontaneous activity there was hardly any delay and the sensitivity to NA in the physiological frequency range (less than 8 Hz) was greatly increased

Another functional aspect of the developing nerve supply is the appearance of an efficient neuronal uptake of NA (*cf* Sachs *et al* 1970) In the adult portal vein this uptake mechanism greatly influences the responses to exogenous NA so that a considerable prejunctional supersensitivity is obtained after denervation (Johansson *et al* 1970) In the present experiments the importance of this mechanism has not been studied in detail but it seems to be a possible explanation for the observed decrease in NA sensitivity after the 4th week (see Fig 7)

The morphological development of the adrenergic innervation has been studied in different

organs of the rat (de Champlain *et al* 1970 Owman Sjöberg and Swedin 1971) However the ontogenesis of the neural supply of the portal vein is not known in detail as yet In the adult rat the functionally important terminal nerve plexus is located between the longitudinal and circular muscle layers (Johansson *et al* 1970 Ljung *et al* 1973) Ts Ao *et al* (1971) found unmyelinated nerve fibers in the adventitia at birth but they did not detect any nerve structures between the two muscle layers in the rat portal vein up to 6 weeks of age The time course for the development of this adult pattern of innervation awaits further study

The hemodynamic consequences resulting from pronounced activity of this single unit type of longitudinally arranged spontaneously active smooth muscle of the portal vein are not known In the previous studies from this laboratory the adult portal vein has been used as an *in vitro* model for vascular smooth muscle of the single unit type i.e. the kind present in the hemodynamically all important precapillary resistance and sphincter vessels Study of the developmental aspects of the young rat portal vein may be important for an understanding of the ontogenesis of single unit vascular smooth muscle in general However it might rather reflect a structural and functional postnatal adaptation to the altered cardiovascular situation occurring in the portal circulatory system after birth or secondary to maternal endocrine influences

In the human fetus the portal vein receives only little blood flow from the inactive gastrointestinal tract There is a direct communication between the umbilical vein and portal sinus which drains into the caval vein i.e. sinus venosus Immediately after birth the umbilical circulation ceases and the umbilical and portal vein blood pressures fall rapidly Several hours later when the gastrointestinal tract begins to function portal vein blood flow increases considerably to cause a marked widening in portal vein diameter during the first 3 weeks of life This increased diameter is associated with an unfolding of longitudinal muscle (Meyer and Lind 1966) In the rat use of the whole body freezing technique has likewise demonstrated that significant increases in the inner diameter of the postnatal portal vein occur at birth and during the first week of life (Marsk 1972)

In experiments on the isolated portal vein from animal species which are more mature than the rat at birth it has been found that spontaneous activity and neurogenic responses appear in the newborn (Stage unpublished) Thus it appears that the postnatal development of the rat portal vein during the first three weeks of life occurs prenatally in other more mature animals Therefore it is likely that the observed vascular maturation is independent of the hemodynamic or endocrine changes at birth

In conclusion a marked structural and functional differentiation occurs in the developing portal vein during the neonatal period of life When this differentiation is complete the portal vein has fully developed a single unit type of longitudinal smooth muscle capable of coordinated phasic contractile activity Once myogenic propagation is established the effectiveness of sympathetic nervous control is greatly enhanced by coordinating the contraction and by recruiting non innervated smooth muscle cells

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## Influence of Amphetamine Sulphate on Cerebral Blood Flow and Metabolism

By

CISTRISTER CARLSSON MAGNUS HAGERDAL AND BO K SIESJO

In the course of a study of the effect of hyperthermia upon cerebral metabolic rate for oxygen ( $CMR_O$ ) and cerebral blood flow (CBF) we have investigated separately other procedures that may increase  $CMR_O$  including administration of amphetamine sulphate. Since the results have disclosed a hitherto unknown effect of amphetamine on CBF and  $CMR_O$  a preliminary account of the results is given.

### Methods

Unstarved male Wistar rats were anesthetized with 2-3% halothane immobilized with tubocurarine chloride ( $0.5 \text{ mg kg}^{-1}$ ) i.v. tracheotomized and ventilated on 70%  $N_2O$  and 30%  $O_2$ . Halothane was withdrawn at the time of tracheotomy. A femoral artery was cannulated for blood pressure recording and for sampling of arterial blood. The superior sagittal sinus was exposed by a small burr hole for sampling of cerebral venous blood. CBF was measured according to the Kety and Schmidt principle using the  $^{133}\text{Xe}$  method described in a previous communication (Norberg and Siesjö 1974).  $CMR_O$  was calculated as the product of CBF and the arteriovenous difference in oxygen content ( $AVD_O$ ). In a few experiments repeated measurements were performed of  $AVD_O$  prior to and following administration of amphetamine. In others cortical blood flow was measured 2 and 5 min after the drug was given using the  $^{14}\text{C}$ -ethanol method described by Eklöf *et al* (1974).

### Results

In all experiments to be reported the body temperature was close to 37°C the  $P_{aCO_2}$  was close to 40 mm Hg and the  $P_{aO_2}$  exceeded 100 mm Hg. In 8 expts CBF and  $CMR_O$  were measured 30 min after the i.v. administration of amphetamine sulphate in a dose of  $5 \text{ mg kg}^{-1}$ . The results were compared to those obtained in 12 un.injected animals. Table 1 shows that there was a highly significant increase in  $CMR_O$  by about 40 per cent. Somewhat unexpectedly CBF increased to an average value of 400 per cent of normal, an increase which cannot be explained by the small increase in blood pressure. In other experiments the  $AVD_O$  was found to decrease to less than 50 per cent of normal already at 1 and 3 min following the i.v. administration of amphetamine in a dose of  $2.5 \text{ mg kg}^{-1}$ . Measurements of regional CBF using the  $^{14}\text{C}$ -ethanol method confirmed that CBF increased about three-fold after 2 and 5 min.

TABLE I Cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMR<sub>O</sub>) in rats 30 min after administration of amphetamine sulphate in a dose of 5 mg kg<sup>-1</sup> i.p. Also given are mean arterial blood pressure (MABP) and arterial P<sub>CO</sub> The values are means  $\pm$  S.E.

Exp. group	MABP	P <sub>a</sub> CO	CBF	AVD <sub>O</sub>	CMR <sub>O</sub>
Control (n=17)	139 $\pm$ 4	37.9 $\pm$ 0.7	110 $\pm$ 4	9.4 $\pm$ 0.4	10.2 $\pm$ 0.2
Amphetamine sulphate (n=8)	163 $\pm$ 3	40.1 $\pm$ 1.0	4.8 $\pm$ 45	3.6 $\pm$ 0.3	14.3 $\pm$ 0.6

$p < 0.001$

The increase in CBF after amphetamine was unrelated to changes in CO<sub>2</sub> tension. Further more studies of tissue metabolites revealed that the tissue contents of lactate and pyruvate remained unchanged. Thus tissue acidosis could not have been responsible for the hyperemia.

### Discussion

Previous observations in man (Abreu *et al.* 1949; Shenkin 1951) have given no indication that amphetamine significantly affects CBF or CMR<sub>O</sub>. Recently Nahorski and Rogers (1973) reported that amphetamine sulphate administered to rats in a dose of 5 mg kg<sup>-1</sup> i.p. caused a decrease in cerebral metabolic flux during the first 30 min period and a subsequent increase thereafter. The method used by the authors was an indirect one and it is not known if it gives a valid measure of metabolic rate under the conditions of the experiments.

The present results demonstrate that CMR<sub>O</sub> increases by about 40 per cent 30 min after i.p. administration of amphetamine sulphate and that there is a severalfold increase in CBF. When CMR<sub>O</sub> is decreased by barbiturate anesthesia or hypothermia or increased by hyperthermia the relative changes in CBF and CMR<sub>O</sub> are similar. Thus the increase in CBF after amphetamine administration is far in excess of the elevation in metabolic rate. This indicates that amphetamine has a more direct effect on the unknown mechanisms that normally adjust the rate of blood flow to the functional activity.

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**$\alpha$ - $\gamma$ -Linkage in the Spinal Generator for Locomotion in the Cat**

By

A SJÖSTRÖM and P ZANGGER

Grillner and Zangger (1974) reported that after a complete transection of all dorsal roots preparations spinalized at lower thoracic or upper lumbar levels can still produce rhythmic locomotor like activity with an alternate activation of flexors and extensors provided that they have been injected with DOPA DOPA and Nialamide Clonidine i.v. or that the transection of the spinal cord is chronic The present study was undertaken to see whether or not  $\gamma$  motoneurons to the appropriate muscles are coactivated with the  $\alpha$  motoneurons during this rhythmic activity

The  $\gamma$  motoneurone activity has been recorded in the form of single  $\gamma$  efferents isolated in peripheral nerve filaments and identified by their conduction velocity from the ventral roots (see Bergmans and Grillner 1969) to either a flexor (*tenuissimus*) or an extensor (*lateral gastrocnemius*) Also muscle spindle afferents (primary endings) identified conventionally (cf Matthews 1972) were recorded from a flexor (anterior tibial) and an extensor (*soleus*) The preparations were decerebrated and spinal (Th 12 to L3) without anesthesia from 2 h prior to the beginning of the recording period All dorsal roots were transected and 50 mg/kg Nialamide was injected i.v. followed by up to 50 mg/kg DOPA After this injection spontaneous rhythmic activity (0.5-1.2 Hz) could occur but during uni- or bilateral continuous stimulation (10-200 Hz) of the cut dorsal roots (L5 or L6) the rhythmic efferent activity increased in frequency and became more regular When recording  $\gamma$ -efferents the preparations were curarized and  $\alpha$  and  $\gamma$  motoneurons were recorded in filaments when recording primary endings a strain gauge was connected to the muscle under investigation The length of the muscle was measured in relation to the maximal length and the corresponding joint angle (Grillner and Udo 1971)

Fig 1 A shows the discharge in a primary ending from a soleus spindle and the marked acceleration during each period of extrafusal contraction For comparison (B) is shown the typical unloading of the ending occurring when the nerve is stimulated with a single shock The graph in D shows the rhythmic increase in discharge frequency in a single extensor  $\gamma$  motoneuron The bars below show that the periods during which the  $\alpha$  motoneurons are accelerated coincide with the increased  $\gamma$  activity Therefore it is no doubt that the results shown in A are due to  $\alpha$ - $\gamma$ -coactivation In C is shown that  $\alpha$  and  $\gamma$  motoneurons to a flexor muscle are also coactivated

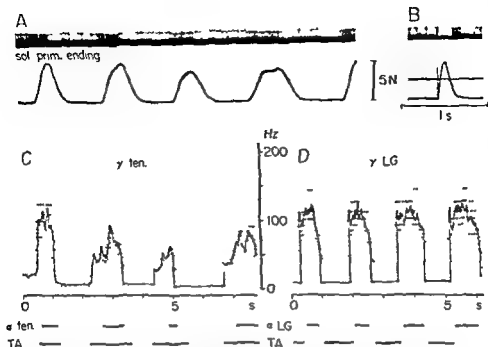


Fig. 1. Rhythmic  $\alpha\gamma$  coactivation to flexors and extensors. *A* shows the activity of a single primary (conduction velocity 85 m/s) ending from an ankle extensor (m. soleus) recorded in a dorsal root filament L 7 (upper trace) during rhythmic isometric contraction of the muscle (tension: lower trace). The muscle was held at a length corresponding to 60° ankle flexion, resulting in a resting discharge of 16 Hz. Continuous bilateral stimulation of L 6 dorsal roots (100 Hz, 60  $\mu$ A square-wave pulses of 0.2 ms duration). In *B* is shown a twitch contraction after stimulation of the muscle nerve obtained just before the recordings in *A*. *C* shows the frequency of discharge (ordinate) in a single flexor  $\gamma$ -efferent (conduction velocity 35 m/s) during spinal stepping recorded from a tenuissimus nerve filament. The recorded sequence was taken just after termination of a period of ipsilateral continuous stimulation of the dorsal root L 6. Dots mark the instantaneous frequency (1/interval length); the curve is plotted with a floating average over the 8 nearest intervals. The horizontal bars below the graph indicate the duration of the activity of a single  $\alpha$ -efferent recorded in the same filament ( $\alpha$  ten.) and the activity in a nerve to an ankle flexor (TA: tibialis anterior). The resolution for the frequency plot was limited by the input device to the computer (digitizer table). *D* shows rhythmic discharges of a single extensor  $\gamma$ -efferent (conduction velocity 29 m/s) recorded in a lateral gastrocnemius (LG) nerve filament during ipsilateral stimulation of L 5 dorsal root (100 Hz, 10  $\mu$ A). Presentation as in *C*. The bars indicate the period of activity in a single  $\alpha$ -efferent recorded in the same nerve filament ( $\alpha$  LG). Note time and tension (100 g.w.t. equals 0.98 N) calibration for *A* and *D*.

Hence it can be concluded that the central spinal mechanism that generates this rhythmic locomotor activity in fact is coactivating  $\alpha$  and  $\gamma$  motoneurons, i.e. there is a central spinal  $\alpha\gamma$  linkage. This is in agreement with the predictions made from the studies of  $\alpha\gamma$  linkage in the late discharges occurring after DOPA (Grillner 1969; see also Purcell 1973). The entire pattern of muscle activity in locomotion can be generated in the low spinal animal (Grillner 1973) and it thus appears that these movements are  $\alpha\gamma$  linked, and it follows that the findings of  $\alpha\gamma$ -coactivation in high decerebrate walking cats should also be due to a spinal mechanism (Severin *et al.* 1967).

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## Brown Adipose Tissue and the Significance of the Venous Plexuses in Pinnipeds

By

ARNOLDUS SCHYTTE BLEX HANS J GRAV and KEITH RONALD

Anatomically the cardiovascular system of Pinnipeds differs in important respects from that of most other mammals in the occurrence of spectacular venous plexuses (Burow 1838). These plexuses (Fig 1 a) which are localized in the neck region on the pericardium on the abdominal wall adjacent to the kidneys and as a stellate renal plexus have been described in detail by Harrison and Tomlinson (1956) and by McCarter (1974). In contrast to this comprehensive anatomical documentation the physiological significance of the plexuses have remained obscure. During recent field studies (in Eastern Canada and off East Greenland) however we have discovered that the venous plexuses of both harp seals and hooded seals appear to be embedded in brown adipose tissue.

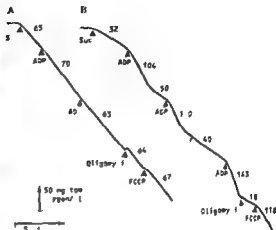
It is generally agreed that brown adipose tissue possesses a decisive thermogenic potential (Smith and Horwitz 1969). When activated the brown adipose tissue produces heat by oxidation of its lipid stores. This oxidation proceeds at a high rate by virtue of mitochondria in which oxygen uptake and energy conservation are loosely coupled processes (Christiansen *et al* 1969). With mitochondrial respiratory control thus suspended this principle allows the tissue to sustain a high rate of respiration and hence of heat production irrespective of whether phosphate acceptor (ADP) is available to the mitochondria.

In separate series of experiments we have confirmed that mitochondria isolated from the brown adipose tissue of harp seals behaved as would be expected of loosely coupled mitochondria (Fig 1 b). The light and electron microscopical appearance of the tissue (Fig 1 c) shows all the features classified by Hull (1966) as typical of mature brown adipocytes. It follows that the harp- and the hooded seals possess functional brown adipose tissue specifically localized to the venous plexuses.

Upon diving the cardiovascular system of the seal is transformed into a heart-brain-lung circuit due to a conspicuous vasoconstriction in almost every tissue except the brain and the heart (Irving 1939). The definite advantage of this redistribution of the blood is that the total oxygen store of blood and lungs is reserved for those tissues which are easily damaged by hypoxia *i.e.* the brain and the heart. The obvious disadvantage of this rearrangement, however is the dramatic decrease in heat production which follows the change from aerobic to anaerobic metabolism in the tissues deprived of circulation. The cooling incurred by this



a



b



c

oxygen  $\text{min}^{-1} \text{mg mitochondrial protein}^{-1}$  In trace B the medium was supplemented with 1 bovine serum albumin. These traces demonstrate that the distinctive property of loose coupling (i.e. respiratory rate independent of the presence of phosphate acceptor) in brown adipose tissue mitochondria can be overcome *in vitro* by fortifying the medium with serum albumin. b Electron micrograph of brown adipose tissue from the pericardial plexus region of a young harp seal pup. A blood vessel is seen adjacent to a brown adipocyte. Note the large concentration of mitochondria adjacent to fat droplets. c Typical laminar mitochondria from the same section.

Fig. 1 a The venous plexus on the pericardium of a harp seal as visualized by angiography during a dive (from Hol *et al.* 1974). b Respiratory pattern of isolated mitochondria prepared from pericardial brown adipose tissue of a 14 day-old harp seal pup according to Gra *et al.* (1970). The oxygen uptake was measured by means of a Clark electrode. The reaction mixtures contained the following final concentrations of components in a total volume of 1.5 ml: 135 mM sucrose, 40 mM N-2-hydroxyethylpiperazine N'-ethane sulphonic acid (HEPES)-buffer pH 7.2, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 33  $\mu\text{M}$  rotenone and mitochondria (0.5 mg mitochondrial protein  $\text{ml}^{-1}$ ). At the points indicated 10 mM succinate (Succ), 67  $\mu\text{M}$  ADP (1/8 oligomycin and 3  $\mu\text{M}$  (trace A) or 50  $\mu\text{M}$  (trace B) carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) were added at a temperature of 5°C. The numbers above each trace represent the specific respiratory rates (ng atoms

deficit in heat production is further reinforced by a maintained or increased blood flow to the poorly insulated brain (Irving 1938). Consequently a marked decrease in deep body temperature is observed in the diving seal (Scholander *et al.* 1942) in spite of increased insulation due to the peripheral vasoconstriction. During such circumstances of negative heat balance warming of the venous return would be of obvious advantage in minimizing cooling of the central body core.

Hol *et al.* (1975) have demonstrated that the venous return is shunted through the venous plexuses during the dive and the present study has focussed attention on the fact that these plexuses are embedded in functional brown adipose tissue. In our opinion this brown adipose tissue-venous plexus complex may function as a high efficiency tubular heat exchange organ when activated. Utilization of the thermogenic potential of the brown adipose tissue would presumably be effected by reflex activation of its sympathetic innervation (Smith and Horwitz 1969).

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## An Attempt to Estimate the *in vivo* Concentrations of Noradrenaline and ATP in Sympathetic Large Dense Core Nerve Vesicles

By

HUGO LAGERCRANTZ GABRIEL FRIED and INGRID DAHLIN

The sympathetic nerve vesicles have generally been assumed to store catecholamines (CA) in the same concentrations and by the same mechanisms as the chromaffin granules from the adrenal medulla. Actually some calculations of the number of vesicles in the nerve terminals have been based on this assumption (Dahlström Haggendal and Hokfelt 1966). The finding of a molar CA/ATP ratio close to 4/1 in nerve vesicle preparations (Schumann 1958) i.e. about the same as in chromaffin granules was thus expected.

However it was surprising to find considerable lower CA content in splenic nerve vesicle preparations apparently consisting to a large part of noradrenaline (NA) storage particles (see Lagercrantz 1971). The low NA concentration in the nerve vesicles from the axons could be due to immaturity and that the vesicles reach their final NA content on arrival to the nerve terminals. This was suggested by Euler (1958) and Stjärne (1968) and further demonstrated by De Potter Chubb and De Schaepdryver (1972) Klein (1973) and Lagercrantz, Kirksey and Klein (1974). Furthermore the finding of low NA contents in the splenic nerve vesicle preparations was to some extent due to NA losses during the critical *post mortem* delay defined as the interval from the time the animals were killed until the nerves could be chilled on ice (Yen Klein and Chen Yen 1973).

The NA/ATP ratio was found to be higher than 4/1 in nerve vesicle preparations when the degree of mitochondrial contamination was considered (De Potter Smith and De Schaepdryver 1970 Lagercrantz and Stjärne 1974). This ratio must also be assumed to be dependent on NA loss during the *post mortem* delay and on whether the nerve vesicles are from the proximal or distal part of the nerve.

The aim of the present report is to calculate the true NA and ATP concentrations in the large dense core vesicles of bovine splenic nerve axons *in situ* and also to try to estimate the concentrations in vesicles in the nerve terminals.

Bovine splenic nerves were chilled on ice in 10 min (15 °C) after killing the animals. Each nerve was divided in one extra- and one intrasplenic section and NA vesicles were isolated from these two sections by differential and density gradient centrifugation on sucrose  $D_2O$  gradients as described earlier (Lagercrantz 1971 Yen *et al.* 1973). The nerve vesicle fraction obtained in the gradients (fraction III) was diluted to isotonicity with hypotonic sucrose solution and centrifuged at 70 000  $g_{90}$  for 30 min to remove most of the mitochondrial contamination (Lagercrantz and Stjärne 1974). The supernatant containing most of the

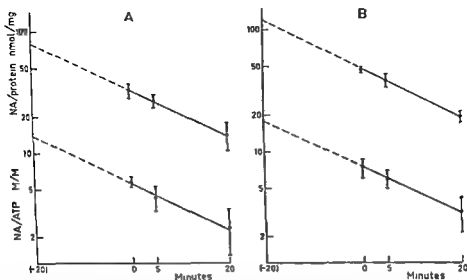


Fig. 1 NA/protein (nmol/mg) and NA/ATP (M/M) ratios in NA vesicle preparations from extra- (A) and intra-splenic (B) nerve segments after incubation at 30°C for 0, 5 and 20 min. Means of at least 4 experiments  $\pm$  S.E. The values are plotted on a semilogarithmic scale with the time of incubation on the abscissa. The duration between killing the animals and chilling the nerves on ice is also indicated on the abscissa (— 0 min) and the ratios are extrapolated back to that moment (dashed lines).

nerve vesicles was incubated at 30°C in the presence of 20 mM K-phosphate buffer pH 7.3 and 10  $\mu$ g/ml Pargyline but without any addition of NA, ATP and  $Mg^{++}$ . Aliquots were removed at the beginning and after 5 and 20 min during the incubation and spun down at  $\approx 30\,000\ g_{max}$  for 30 min.

Analyses (for details see Lagercrantz 1971). The pellets were extracted in 1 ml of 0.4 M perchloric acid for 10 min then 1 ml of distilled water was added. After centrifugation 1 ml of the supernatant was used for determination of NA by the trihydroxyindole method and the rest of the supernatant was neutralized to pH 7.0–7.4 with KOH and Tris-buffer. The perchlorate precipitate was carefully removed by centrifugation before storage in the freezer. The ATP assay was performed by a modified fire fly method. Various amounts of ATP were added to some samples as internal standards. The recovery of ATP was on average 80 per cent. The ATP values were corrected for the losses. The first obtained pellet was used for protein assay by the Folin method.

The results are shown in Fig. 1. The NA/protein ratio was found to be  $32.6 \pm 2.4$  ( $n=7$  S.E.) nmol/mg protein in the proximal nerve segment preparation while  $45.8 \pm 2.4$  ( $n=7$ ) in the distal one ( $p<0.01$ ). The NA/ATP ratio was found to be  $5.7 \pm 0.5$  mol/mol ( $n=10$ ) in the proximal segment preparation and  $7.4 \pm 1.2$  ( $n=8$ ) in the distal one ( $p<0.05$ ). The NA/protein ratio decreased to 50 per cent in about 16 min and the NA/ATP ratio in about the same time. About 15 per cent of the ATP content and 16 per cent of the proteins were released during the incubation (20 min).

In order to get an idea of the *in vivo* concentrations of NA and ATP extrapolation was performed from the points obtained at different time intervals during the incubation to the estimated zero time when the animals were killed. The release rate of NA and ATP during the *post mortem* delay at the slaughter house was found to correspond fairly well with the release rate during incubation at 30°C (to be published). This was also tested by leaving the nerves at the warm spleens for 20 min before they were chilled on ice (Yen *et al.* 1973).



ATP was found to be released slowly compared with NA in accordance with earlier observations (Euler, Lishajko and Stjärne 1963 and Stjärne 1964) and not at all released with NA in the stoichiometric ratio 1/4 as from the isolated chromaffin granules (Stjärne 1964). The NA/ATP ratio is calculated to be about 12 in the proximal nerve vesicles and 18-19 in the distal ones.

The NA/protein and NA/ATP ratios in the nerve terminal vesicles cannot be determined directly but by comparison with the increase of the NA/dopamine  $\beta$ -hydroxylase ratio in vesicle preparation from the proximal nerve segment and a crude vesicle preparation from the spleen the NA/protein ratio can be assumed to be 400-500 nmol/mg and the NA/ATP ratio about 50/1 (cf. Lagercrantz *et al.* 1974).

**Conclusions:** The *in vivo* amine concentration expressed as nmol NA/mg protein was estimated to be 80-120 and 400-500 in the large dense core nerve vesicles from the distal and proximal segment and the spleen respectively. The NA/ATP ratios (M/M) were estimated to be 12/1, 18-19/1 and in the range of about 50/1 in the corresponding nerve vesicle preparations.

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## Dual Adrenoceptor-mediated Control of Noradrenaline Secretion from Human Vasoconstrictor Nerves Facilitation by $\beta$ -receptors and Inhibition by $\alpha$ -receptors

By

LENNART STJÄRNE AND JAN BRUNDIN

Previous work with isolated superfused strips of human blood vessels has provided evidence that vasoconstrictor nerves in man possess neural  $\alpha$  adrenoceptors capable of mediating negative feedback control of the secretion of noradrenaline (NA) (Stjärne and Gripe 1973). The present study was prompted by reports based on work with different tissues and species that sympathetic nerves may in addition possess  $\beta$ -adrenoceptors enhancing NA secretion and thus capable of mediating positive feedback control of this process (Langer *et al* 1974 Stjärne 1975 a). The aim of the present work was to examine the possible role of  $\beta$ -adrenoceptors in the control of the secretion of NA from human vasoconstrictor nerves.

Isolated strips of omental arteries and veins obtained from 8 female patients aged 26 to 95 years and undergoing abdominal surgery under various forms of routine general anesthesia were used for the experiments. Premedication always included atropine 0.5 mg s.c.

The methods used for setting up superfused field stimulated strips of arteries and veins have been explained in detail elsewhere (Stjärne and Gripe 1973 Stjärne and Brundin 1975). The tissues were preincubated with  $10 \mu\text{Ci}$  (260 ng) H(-)-NA (New England Nuclear Corp.) and  $0.6 \mu\text{M}$  desipramine and  $10 \mu\text{M}$  normetanephrine were added to the superfusion medium (Tyrode solution aerated with 6.5%  $\text{CO}_2$  in  $\text{O}_2$ , temperature was 31°C and flow rate  $2.5 \text{ ml min}^{-1}$ ) to prevent rebinding of NA. The secretion of NA evoked by field stimulation of the vasoconstrictor nerves with trains of 300 shocks at 1 Hz (biphasic pul = 1.5 ms in duration applied voltage on Grass S44 stimulator 90 V) was monitored by measuring  $\Delta t$  the fractional secretion of H-NA per applied shock (Stjärne and Gripe 1973 Stjärne and Brundin 1975). The value for  $\Delta t$  in each experiment was normalized in relation to the average obtained during the first two control stimulations in that same experiment which was given the value of 1 relative unit.

The obtained value for  $\Delta t$  per shock during the initial control period was  $(4.09 \pm 0.21) \cdot 10^{-4}$  ( $n = 40$ ) which is close to previous observations (Stjärne and Gripe 1973 Stjärne and Brundin 1975).

In agreement with the above mentioned finding in isolated cat and guinea pig tissues

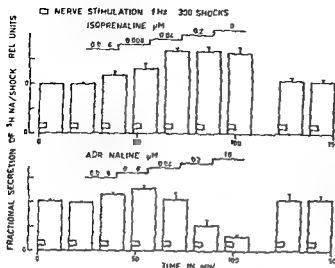


Fig. 1 Fractional secretion of  $^3\text{H}$  NA per shock ( $\Delta t$ ) from strips of human omental arteries and veins (pooled data relative units of Text). Effects of isoprenaline (4 expts) and of adrenaline (7 expts). Means  $\pm$  S.E.

(Langer *et al* 1974, Sjöarne 1975a) isoprenaline (L Isoproterenol D bitartrate Sigma) enhanced  $\Delta t$  ( $p < 0.001$ ). The effect was seen already at  $0.0016 \mu\text{M}$  and increased with the concentration to reach its maximum a rise of  $67.7 \pm 6.2\%$  ( $n = 4$ ) at  $0.04 \mu\text{M}$  (Fig. 1). This shows that human vasoconstrictor nerves possess extremely sensitive  $\beta$  adrenoceptors capable of markedly enhancing the amount of NA secreted per applied shock.

According to Langer *et al* (1974) the secretion of NA from sympathetic nerves in cat and guinea pig tissues is depressed by the  $\beta$  antagonist propranolol  $0.1 \mu\text{M}$ . Their interpretation was that the secretion of NA from sympathetic nerves is in part dependent on  $\beta$ -adrenoceptor mediated positive feedback control.

However propranolol  $0.1$ – $1 \mu\text{M}$  had no marked effect on  $\Delta t$  in the present study.  $\beta$  adrenoceptor mediated positive feedback mechanisms thus do not seem to be indispensable or even quantitatively important for the basic processes of NA secretion from human vasoconstrictor nerves. Some clue as to the possible physiological function of the extremely sensitive neural  $\beta$ -adrenoceptors may be obtained from the finding that (–)-adrenaline (A) at concentrations known to occur in peripheral plasma in man during enhanced adrenomedullary secretion (Vendssalu 1960) significantly ( $p < 0.001$ ) enhanced the secretion of H NA (Fig. 1). At  $0.0016 \mu\text{M}$  the rise was  $15.0 \pm 3.0\%$  ( $n = 7$ ) and at  $0.008 \mu\text{M}$   $26.2 \pm 7.7\%$  ( $n = 7$ ). At higher concentrations A depressed  $\Delta t$  presumably via the less sensitive  $\alpha$  adrenoceptor mediated control mechanisms (Sjöarne and Gripe 1973).

The present findings show that the secretion of NA from human vasoconstrictor nerves may be controlled by a dual set of adrenoceptors with antagonistic effects on the secretory process. The low sensitivity of the  $\alpha$  adrenoceptors suggests that they can only be triggered by NA at the high concentration occurring in synaptic clefts and that their rôle is to exert negative feedback control of NA secretion. The extremely high sensitivity of the  $\beta$ -adrenoceptors which should enable them to detect circulating catecholamines suggests that they may subserve the function of enhancing the secretion of sympathetic neurotransmitter during conditions of increased secretion of adreno-medullary hormone (Sjöarne 1975b).

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## Effects of 7-Hydroxy- $\Delta^6$ -Tetrahydrocannabinol and Some Related Cannabinoids on the Guinea Pig Isolated Ileum

By

SUNE ROSELL AND STIG AGURELL

Since the structure of  $\Delta^1$  Tetrahydrocannabinol ( $\Delta^1$  THC) was elucidated by Gaoni and Mechoulam (1964) some 15 other cannabinoids have been isolated. Both  $\Delta^1$  THC and monohydroxylated metabolites are found in mouse brain following intravenous injection of  $\Delta^1$  THC (*cf* Jones *et al* 1974) and it has been suggested that the CNS activity of  $\Delta^1$  THC is mediated wholly or in part by the monohydroxylated compounds (Grunfeld and Edery 1969, Ben Zvi, Mechoulam and Burstein 1970, Mechoulam 1970, Christensen *et al* 1971, Lemberger *et al* 1972, 1973).  $\Delta^1$  THC is about as active as  $\Delta^8$  THC (Edery *et al* 1972) and appears to have similar psychological effects in man (Hollister and Gillespie 1971).

To investigate the mechanisms of action exerted by the psychoactive cannabinoids, isolated peripheral tissues may serve as test objects provided that there is a close relationship between the behavioural actions and the effects on the isolated organ. In addition the cannabinoids should be active in concentrations which produce the behavioural effects. The present investigation indicates that the isolated guinea pig ileum may be suitable as such a test object.

We have studied the effects of 4 cannabinoids:  $\Delta^8$  tetrahydrocannabinol ( $\Delta^8$  THC), 7-hydroxy  $\Delta^6$  tetrahydrocannabinol (7-OH  $\Delta^6$  THC), cannabidiol (CBD) and cannabinol (CBN) on the guinea pig isolated ileum. The substances were dissolved in ethanol which in the concentrations used had no effects. The terminal portion was used after the 5 cm nearest to the ileo-caecal junction had been discarded. The isolated ileum was suspended in Krebs solution (5 ml) bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The temperature was 36-37°C. Rectangular electrical pulses of 0.5 ms duration, supramaximal strength (50 V) and 0.1 Hz were applied to the electrodes. Isometric or isotonic contractions were recorded by means of transducers (Grass FT 03 and Harvard Apparatus respectively) and displayed on a Rika Denki ink writer.

7-OH  $\Delta^6$  THC reduced the twitch response at a concentration in the bath of approximately 1 ng/ml or higher (Fig. 1). The effect was noted within 2 min and was maximal within 10-15 min. Despite repeated washings the twitch inhibition persisted for about an hour following 1-5 ng/ml. On account of the high lipid solubility of the cannabinoids this may not be unexpected. Due to the long duration of action it was difficult to establish an accurate threshold dose and dose response curves. In some experiments we stimulated the ileum with acetylcholine, histamine or 5-hydroxytryptamine (5-HT). 7-OH  $\Delta^6$  THC reduced the

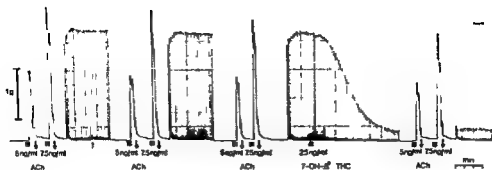


Fig 1 Effect of 7 OH  $\Delta^9$  THC 25 ng/ml on twitches and acetylcholine responses of guinea pig ileum. Acetylcholine was added (■) to the bath while stimulation was temporarily stopped. Washings indicated by ↓

twitches evoked by 5 HT but those evoked by acetylcholine or histamine were not at all or very little affected (Fig 1). These data indicate that the inhibition of the twitch is of presynaptic origin. Both histamine and acetylcholine act directly on the smooth muscle whereas 5 HT primarily acts by stimulating the nervous structures of the intestinal wall (Rocha e Silva, Valle and Picarelli 1953).

$\Delta^9$  THC also inhibited the electrically induced twitches but was approximately 10 times less potent than 7 OH  $\Delta^9$  THC. Furthermore the inhibition was much more gradual in onset (Fig 2). CBD and CBN were without effect at concentrations up to 100 ng/ml as also was found by Layman and Milton (1971).

The quantitative contribution of the 7 hydroxylated metabolites of  $\Delta^9$  and  $\Delta^8$  THC towards the psychopharmacological activity of THC in man is still unresolved. However the 7 hydroxylated compounds are more or at least as active as their non hydroxylated analogues (cf Gill, Jones and Lawrence 1973; Jones *et al* 1974; Brande and Szara 1975).

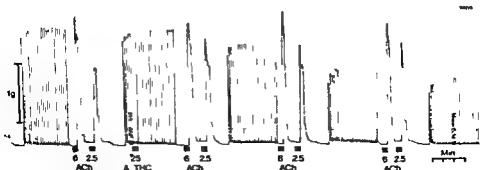


Fig 2 Effect of  $\Delta^9$  THC 5 ng/ml on twitches and acetylcholine responses of guinea pig ileum. The numbers indicate concentrations in the bath in ng/ml.

CBD and CBN are thought to be much less active (Edery 1972). This order of potency is in agreement with the present results. In addition 7 OH  $\Delta^9$  THC is effective on the guinea pig ileum in very low concentrations comparable to those that may be achieved while smoking cannabis. Thus cannabis smokers may absorb 1–10 mg of  $\Delta^9$  THC to achieve psychic effects (Brande and Szara 1975). If this amount is evenly distributed in the whole body the concentration of  $\Delta^9$  THC and its metabolites would be in the order of 50 ng/ml. Thus the isolated guinea pig ileum is affected by 7 OH  $\Delta^9$  THC in concentrations which may have psychoactive effects in the human.

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## Regional and Single Glomerular Blood Flow in the Rat Kidney Prepared for Micropuncture A Methodological Study

By

Ö KALLSKOG L O LINDBOM H R ULFENDAHL and M WOLGAST

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### Abstract

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KALLSKOG Ö L O LINDBOM H R ULFENDAHL and M WOLGAST *Regional and single glomerular blood flow in the rat kidney prepared for micropuncture A methodological study* Acta physiol scand 1975 94 145-153

Regional renal and single glomerular blood flow was investigated in rats using  $^{141}\text{Ce}$  and  $^{85}\text{Sr}$  labelled microspheres. The kidneys were prepared for micropuncture and also the artery was catheterized with a steel cannula in order to make injections of  $^{133}\text{Xe}$  and vasoactive substances selectively into one kidney. The microspheres were first separated by sedimentation into a narrow fraction and then injected as a plasma suspension into the carotid artery. The two batches were infused at half an hour intervals. Single glomeruli were sampled from kidney sections with the aid of silicon rubber casts allowing the identification of the glomeruli both with respect to their localisation and the postglomerular appearance. The results showed a relatively high blood flow in the superficial and juxtamedullary glomeruli of about 100 ml/min per 100 g rat whereas in the other glomeruli the blood flow was about 70 ml/min per 100 g rat. No differences could be observed between the right untouched kidney and the left prepared for micropuncture. Furthermore no discrepancies between the results from the two injections could be found. The data on the total renal blood flow obtained by the  $^{133}\text{Xe}$  wash-out method agreed well with the microsphere method.

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During recent years haemodynamic conditions within the renal microvasculature have been the object of keen interest especially in regard to the renal handling of salt and water. Thus the formation of primary urine is governed not only by the hydrostatic and oncotic forces in the glomerular capillaries but also by the glomerular plasma flow (Brenner *et al* 1972, Deen *et al* 1972). The tubular fluid reabsorption is influenced by the same driving forces operative in the peritubular capillary network (Levy and Windhager 1968, Brenner and Berliner 1969, Fackhuik *et al* 1971, Persson *et al* 1972). The study on renal tubular handling of salt and water therefore necessitates investigations of the renal circulatory conditions in addition to studies on the tubular transport kinetics. For this purpose the microsphere method (as modified by the use of spheres in a narrow size range (Kallskog *et al* 1972)) has been used to study the regional renal cortical as well as the single glomerular blood flow in rats on animals prepared for micropuncture in the usual manner. The aim of this investigation has been (1) to estimate the normal flow pattern within different glomerular popula-



tions and (2) to test the reproducibility by two consecutive injections of microspheres and (3) to compare the results from the right untouched kidney with the left prepared kidney. The latter was exposed via a flank incision, free dissected and suspended into a lucite cup. The left renal artery was then catheterized in order to measure the renal blood flow with the  $^{133}\text{Xe}$  wash-out method and also to permit the administration of vasoactive drugs selectively into one kidney.

The glomerular blood flow was calculated as the mean from 50 glomeruli. The glomerular populations were not only classified according to their localisation within the renal cortex but also with respect to their postglomerular appearance, permitting studies on the true juxtamedullary glomeruli. The results indicate a comparatively high glomerular perfusion in both the very superficial and in the juxtamedullary glomeruli. Only minor differences in the values from the right control and the left kidney could be observed, thereby indicating that the preparation for micropuncture and renal artery catheterization was accompanied with only a slight disturbance at least as suggested by the circulatory variables studied. Furthermore, there was no significant difference between the results from the first and the second microsphere injection. The total renal blood flow calculated from the microsphere method agreed well with those obtained by the  $^{133}\text{Xe}$  wash out method, even though the interpretation from the latter technique in terms of regional renal blood flow (compartmental analysis) can be difficult.

### Material and Methods

All experiments were performed on male Sprague-Dawley rats weighing between 246 and 311 g. The animals were anaesthetized with Inactin® (Chemical Fabrik Promonta GmbH, Hamburg, West Germany) intraperitoneally (120 mg/kg B.W.). A tracheotomy was performed and placed on a servo-controlled heating pad. Both femoral arteries were catheterized using polyethylene catheters. The left femoral artery was used for blood pressure recordings and the right artery for continuous sampling of blood during the injection of the microspheres. The right carotid artery was catheterized with a relatively thick (1 mm) catheter with the tip placed in the aortic root just above the aortic valves. The left kidney was exposed via a flank incision and suspended into a lucite cup. For renal artery cannulation, a lumbar artery located just opposite to the left renal artery was free-dissected and tied off. A steel cannula with an outer diameter of 200  $\mu\text{m}$  and formed appropriately was then introduced into the lumbar artery and passed across the aorta into the renal artery. With this technique, no free dissection of the renal artery or the hilus region was necessary. No visible change in the kidney surface could be observed during the catheterization. After completion of the surgery, the rats were allowed to rest for approximately one hour.

In the present series,  $^{141}\text{Ce}$  and  $^{85}\text{Sr}$  (3M Co., Saint Paul, Minn., USA) labelled 15  $\mu\text{m}$  microspheres were used. The spheres were first subjected to ultrasonic agitation and then separated by a sedimentation procedure in large glass cylinders (according to Källskog *et al.* 1972). Thus, the size range was reduced to about half of the original  $+5 \mu\text{m}$  ( $\pm \text{SD}$ ). 400 000 spheres were used for each injection—the injection time was 70 s. At the same time, the suction pump connected to the femoral artery catheter was running with a sampling rate of 0.6 ml/min. The suction was disrupted some 15 s after the completion of the microsphere injection. The second injection of spheres was made 1/2 h after the first one.

The renal vascular network was then filled with a silicone rubber compound (Microfil, Canton, Bomed Prod. Inc., Box 2017, Boulder, Colorado 80307, USA) in an aortic catheter (Bankir *et al.* 1973). The kidneys were then excised together with some other organs such as the brain, the heart, the lungs, the stomach, the liver, the spleen, the suprarenal glands, pieces of muscular and adipose tissue, etc. and weighed. The kidneys were macerated for 30 min in 5 N HCl at 40°C, embedded in carboxymethyl cellulose gel and frozen to  $-20^\circ\text{C}$ . With a microtome, 0.01 mm thick sections were cut in the longitudinal direction of the kidney (see Fig. 1). From these sections, glomeruli were sampled manually with the guidance of a microscope with 10 times magnification. The glomeruli were subdivided with respect to their localisation: (a) superficial glomeruli (type I), (b) subcortical glomeruli (II), (c) deep glomeruli without vasa recta (IIIa) and (d)

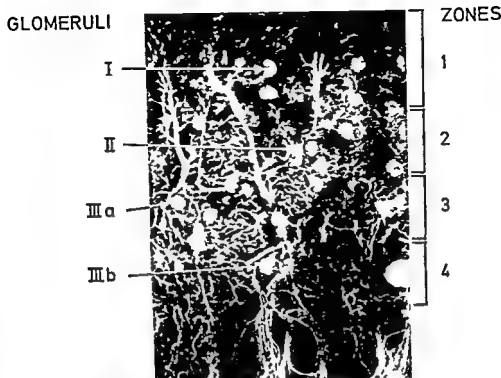


Fig. 1 Microphotograph of a rat kidney section showing the subdivision into different zones (to the right) and the different glomerular populations studied (to the left)

glomeruli which by microdissection were found to empty into vasa recta (III b)—the so-called true juxtamedullary glomeruli (see Fig. 1). It should be emphasized that these latter glomeruli mostly were located in the juxtamedullary zone but also not rarely in the middle of the cortical parenchyma. At least 50 glomeruli of each type were sampled. After the sampling the cortical part of the sections were subdivided into four zones according to Fig. 1. Zone 1 will then constitute the superficial cortex, zones 2 and 3 the mid-cortical area and zone 4 the juxtamedullary region. Effort was made to make the zones representative for the corresponding parts of the whole kidney. The blood and tissue samples were analyzed with respect to  $^{141}\text{Ce}$  and  $^{85}\text{Sr}$  content in a gamma spectrophotometer (Nucab AB 100 039 S-4 10, Västra Frölunda, Sweden). The total amount of activity injected was determined from the activity obtained from the injection syringe before and after the injection of the microspheres. The blood flow to the specimen was calculated according to  $f = M/V_b$ , where  $f$  is the blood flow,  $M$  the activity in the specimen in question and  $V_b$  the activity found in the blood sample. Cardiac output (or more correctly the cardiac output minus the coronary blood flow) was determined by the same equation but the factor  $M$  will denote the total amount of activity injected and  $f$  will represent the cardiac output.

In some experiments the renal blood flow was investigated using the  $^{133}\text{Xe}$  wash-out technique in order to make repeated determinations. For this purpose about  $10 \mu\text{Ci}$  of  $^{133}\text{Xe}$  in  $50 \mu\text{l}$  of saline was rapidly injected into the renal artery catheter. The counting rate was recorded by a gamma detector with the collimator focused on the kidney. The blood flow was determined from the slope of the wash-out curve (according to Thorburn *et al.* 1963).

## Results

The results from the experiments are depicted in Table I. Some of the variables are also shown in Fig. 2 and 3. The cardiac output was calculated as  $32.7 \text{ ml/min per } 100 \text{ g rat}$  decreases

TABLE I Data on systemic and renal circulatory parameters obtained from 2 consecutive microsphere injections. The results from the two injections showed no significant difference in any of the variables ( $p > 0.1$  or more). Likewise there was no difference between the right untouched and the left kidney prepared for micropuncture ( $p > 0.1$  or more in all instances)

										Mean $\pm$ S D
Rat weight g		246	254	284	284	293	309	311 <sup>a</sup>	292	
Perfusion pressure mm Hg		Contr 113	111	115	109	100	117	130	117	111.7 $\pm$ 5.9
		Exp 107	107	115	107	96	118	133	115	109.3 $\pm$ 7.4
Cardiac output ml/min 100 g rat		Contr 45	33	37	32	26	27	39	29	32.7 $\pm$ 6.6
		Exp 40	31	35	29	25	29	38	27	31.1 $\pm$ 5.2
RBF left kidney ml/min 100 g rat		Contr 245	214	227	204	153	221	229		180.2 $\pm$ 0.31
		Exp 256	243	227	178	158	266	319		164.2 $\pm$ 0.45
Blood flow cortical zones left kidney $\mu$ l/min	1	Contr 280.3	142.0	185.6	156.1	81.7	118.9	117.1	114.8	
		Exp 218.8	130.0	207.7	128.5	78.4	149.3	132.2	113.1	
	2	Contr 154.5	107.1	71.3	124.0	109.5	120.0	164.9	67.5	
		Exp 131.9	144.4	72.5	113.2	107.4	158.7	180.0	67.5	
	3	Contr 122.3	87.9	61.7	93.1	78.3	89.0	78.4	56.4	
		Exp 118.3	133.1	58.4	90.7	80.3	111.2	91.3	59.7	
	4	Contr 108.7	64.5	61.9	61.1	77.5	108.9	138.5	59.7	
		Exp 113.9	110.3	56.6	69.1	85.3	141.2	125.9	72.4	
Glomerular blood flow left kidney ml/min 100 g rat	I	Contr 154.3	113.6	143	116.0	62.0	109.7	276.5	178.0	122.6 $\pm$ 36.6
		Exp 119.0	125.8	154.9	103.3	73.4	157.5	175.8	114.3	121.2 $\pm$ 29.7
	II	Contr 95.2	40.4	77.3	76.1	35.4	96.7	83.5	62.1	68.3 $\pm$ 74.2
		Exp 86.5	65.7	87.2	68.2	55.8	113.2	84.1	85.6	80.2 $\pm$ 19.0
	III a	Contr 60.6	36.6	65.4	61.3	59.0	60.9	118.5	61.5	65.1 $\pm$ 9.8
		Exp 68.6	95.8	46.9	66.0	44.4	74.9	115.7	52.5	64.7 $\pm$ 18.1
	III b	Contr 102.0	69.0	123.6	111.8	70.4	93.7	166.4	106.4	96.7 $\pm$ 70.6
		Exp 98.0	91.0	82.3	125.1	47.4	119.8	143.3	96.0	94.2 $\pm$ 25.7
RBF right kidney ml/min 100 g rat		Contr 289	274	198	180	165	224	273		177.2 $\pm$ 0.49
		Exp 294	306	185	173	162	236	252		168.7 $\pm$ 0.61
Blood flow in cortical zones right kidney $\mu$ l/min	1	Contr 165.0	254.9	233.3	138.8	97.8	139.5	68.0	147.6	
		Exp 143.8	230.3	191.1	101.1	88.3	163.8	25.2	115.5	
	2	Contr 130.7	162.1	99.7	134.9	48.7	109.9	115.4	92.4	
		Exp 97.5	166.4	96.6	110.0	57.0	147.9	123.5	99.9	
	3	Contr 72.1	81.9	59.4	75.9	36.1	74.9	82.4	47.4	
		Exp 65.7	104.7	57.3	81.7	37.1	85.7	84.8	57.0	
	4	Contr 111.1	104.1	90.6	106.3	38.1	101.6	71.4	88.4	
		Exp 85.9	113.6	89.7	106.3	45.3	135.9	46.6	39.1	
Glomerular blood flow right kidney ml/min 100 g rat	I	Contr 137.5	151.0	193.4	116.8	110.3	168.1	259.0	146.9	146.3 $\pm$ 78.8
		Exp 150.4	138.8	148.1	118.9	112.1	174.4	236.1	109.7	136.2 $\pm$ 57.7
	II	Contr 74.1	66.3	68.9	76.0	58.1	93.5	175.6	45.0	68.8 $\pm$ 15.7
		Exp 66.0	87.2	46.1	57.0	71.1	96.5	108.9	51.3	67.9 $\pm$ 18.6
	III a	Contr 93.1	46.6	45.4	52.8	55.1	62.9	60.0	76.1	54.7 $\pm$ 0.6
		Exp 75.4	69.4	51.9	56.3	51.3	67.4	16.9	30.9	57.5 $\pm$ 14.9
	III b	Contr 106.4	135.6	97.3	173.4	71.4	101	120.0	55.4	99.0 $\pm$ 27.9
		Exp 110.9	141.9	69.7	102.0	75.4	112.2	116.1	76.3	98.3 $\pm$ 76.1

<sup>a</sup> This experiment is excluded in the statistics

ing insignificantly to 31.1 ml/min in the second injection. The scattering of the values was moderate: standard deviations were calculated at 7 and 5 ml/min per 100 g rat respectively. The left kidney blood flow was  $2.1 \pm 0.3$  ml/min per 100 g rat (mean  $\pm$  S D) and showed no change during the second microsphere injection. Furthermore there was no significant difference between the experimental left kidney and the right untouched kidney: the blood flow was almost exactly the same in the two as was the scattering. The results on the glomerular blood flow pattern is shown in Table I and also in Fig. 2. The blood flow in the superficial glomeruli is then clearly higher than in any other glomerular population amounting to 120

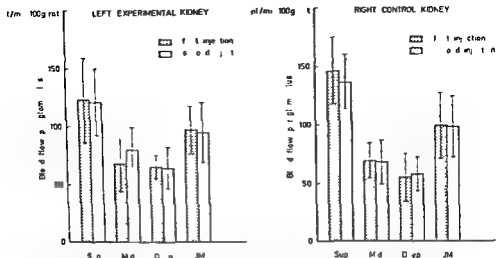


Fig. 2. Glomerular blood flow in ml/min and 100 g rat for superficial (I) midcortical (II) deep cortical (III a) and true juxtamedullary (III b) glomeruli. The pattern of distribution with a higher blood flow in glomeruli types I and III b in relation to types II and III a is highly significant and is valid for both kidneys and for both the first and the second injection.

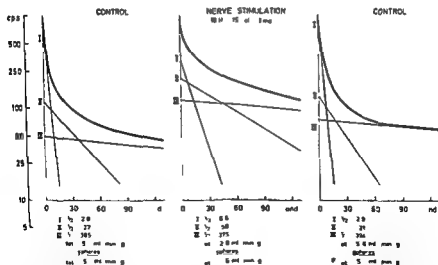


Fig. 3. The figure shows the curves obtained from three injections of  $^{133}\text{Xe}$  into the renal artery catheter of one rat. The first curve (to the left) was obtained during normal control conditions, the second during stimulation of the renal nerves and the third again during normal control conditions. The first injection was preceded by two microsphere injections made during control and during renal nerve stimulation respectively. The Xenon wash-out curves have been subdivided into 3 semilogarithmic components. The total renal blood flow was calculated from the weighted mean of components I and II assuming a partition coefficient of 0.7. The resulting flow would then refer to more than 90% of the total renal blood flow.

TABLE II Student's *t* test for the comparison of glomerular blood flow rates. The table shows that the blood flow to the superficial and juxtamedullary glomeruli (types I and III b) is significantly higher than that to the glomeruli types II and III a. The difference between the superficial (I) and the juxtamedullary (III b) glomeruli was barely significant. Comparing the midcortical glomeruli (II) with deep cortical glomeruli (III a) showed clearly no significant difference.

	Glomerular blood flow first injection						Glomerular blood flow second injection					
	I-II	I-III <sub>a</sub>	I-III <sub>b</sub>	II-III <sub>a</sub>	II-III <sub>b</sub>	III <sub>a</sub> -III <sub>b</sub>	I-II	I-III <sub>a</sub>	I-III <sub>b</sub>	II-III <sub>a</sub>	II-III <sub>b</sub>	III <sub>a</sub> -III <sub>b</sub>
Left kidney	<0.01	<0.01	0.1	<0.5	<0.05	<0.01	<0.01	<0.01	<0.1	>0.1	>0.1	<0.05
Right kidney	<0.01	<0.01	<0.01	>0.1	<0.05	<0.01	<0.01	<0.01	<0.02	>0.1	<0.05	<0.01

ml/min per 100 g rat. The difference between the left and the right kidney is fairly moderate and furthermore there are only small discrepancies between the results from the first and second injection. In the deeper glomeruli (II and III a see above) the blood flow was considerably less of about 70 ml/min per 100 g rat. The pattern of high blood flow in the superficial and juxtamedullary glomeruli compared to the midcortical ones is characteristic and highly significant (see Table II).

The results from the slices given in Table I support the conclusions made above with only a slight difference between the first and second injections.

Fig. 3 gives the curves obtained from a typical experiment with the 133 Xenon wash-out method during control conditions—after renal nerve stimulation and a new control determination. The total renal blood flow was calculated as the weighed mean of compartment I and II to which more than 90% of the total renal blood flow is diverted. The last component (III) was not utilized not because of the difficulties in determining the blood flow but due to the hazard in estimating the 'volume of distribution' and its translation into a real volume or weight. The former will be influenced by the recirculating indicator and the setting of the baseline and the latter by the difficulties in estimating the partition coefficient (see Thorburn *et al.* 1963, Ladefoged *et al.* 1965). The results from the two microsphere injections carried out just before the last control study by the 133 Xenon injection are also shown in the figure. As was the case in all other studies the results from the two methods agreed.

### Discussion

The prerequisite for the use of the microsphere method in the determination of regional blood flow in an organ is that the spheres will be distributed in exact proportion to the blood flow and that they all will be trapped in the capillary bed. This is especially pertinent with respect to the renal circulation where it has been suggested that axial streaming of the red cells and thereby also of the microspheres in the interlobular arteries would leave a cell poor fraction of blood to perfuse the juxtamedullary glomeruli while the superficial structures should be perfused with a cell rich fraction (Pappenheimer and Winter 1955). The magnitude of this cell separation however seems indeed to be small. Ulfendahl (1962) found that the haematocrit value in the superficial 'venae stellatae' of the cat kidney was only about

10 per cent larger than that in the central arterial blood. This argument does not rule out the possibility that large microspheres (with a large amount of radioactivity) could escape trapping in the deeper cortical regions and then by necessity would be diverted to the more superficial structures. This possibility was considered by Kallskog *et al* (1972) showing that the blood flow calculated from the amount of radioactivity trapped agreed well with the relative blood flow calculated from the number of spheres trapped. Furthermore Bankir *et al* (1973) in an extensive study on single glomeruli perfusion in the rabbit kidney were able to show that the size distribution of the spheres trapped into the different glomerular populations was identical with the distribution in the batch injected. This is also supported by the observations of McNay and Abe (1970) showing the same distribution for spheres varying in size from 18 to 36  $\mu\text{m}$ . The density of the spheres would not play a large role as essentially equal distribution was obtained from ceramic spheres with a weight of 3 g/ml as for 3M microspheres weighing about 1.5 g/ml. A different opinion however was reached by Wallin *et al* (1970) on comparing the regional renal cortical flow pattern in dogs with labelled antibodies and labelled microspheres. The two methods corroborated in all the cortical regions except for the very superficial parts where the microsphere method in most instances gave a higher blood flow value. This would be compatible with the concept of axial streaming of the more heavy 3M microspheres. This phenomenon could at least partly be explained if unseparated spheres were used where the large spheres and aggregates would be trapped in the interlobular arteries. However during volume expansion the two methods agreed. In the rat Wallin *et al* (1971 b) found the same pattern of distribution for the two methods during antidiuresis whereas during saline expansion a moderate but significant difference could be observed.

Besides systematical errors the scatter can be expected to be large in determinations of small blood flows which obviously is a limitation in studying small changes. This scatter is to a great part inherent in the analytical method itself: the standard deviation is not equal to the square root of the number of counts analysed but rather to the square root of the number of spheres trapped. In the present paper the number of spheres totally injected was chosen so that 1 to 2 spheres should be trapped in each glomerulum. As about 50 glomeruli from each population were sampled the variability in the blood flow values ought to be as high as 10–15 per cent. A further contribution to this scatter is the variability in the size of the microspheres as the amount of radioactivity per sphere is proportional to the weight of the sphere. In our experience the original batch will include large spheres as well as aggregates of spheres which then could cause a variability almost invalidating the method. In conclusion measurement of blood flow in small vascular units more or less necessitates the use of spheres in a narrow size range. But even so the variability will most likely be about 20%—as found in the present series.

The results from this investigation show an almost complete agreement with that obtained by Bankir *et al* (1973) in experiments on rabbits using the same microsphere technique and the same identification of glomeruli with the aid of silicon rubber casts. The most striking feature is the higher blood perfusion in the superficial and the juxtamedullary glomeruli. These results however are compatible with the study of Kallskog *et al* (1974) in which the superficial single glomerular filtration rate was 15–20 nl/min per 100  $\mu\text{m}^2$  rat on the same strain.

of Sprague Dawley rats. This figure should be related to an average single glomerular filtration rate of about 12 nl/min per 100 g rat calculated 1) from the total renal filtration and 2) under the assumption that the kidney will contain 30 000 glomeruli. It should be noted in this context that these values are somewhat different from other investigations (see Brenner and Daugharty 1972, Schnermann 1972, de Rouffignac and Bonvalet 1972).

The majority of the glomeruli in the kidney will however belong to groups II and III where the blood flow was estimated at about 70 nl/min per 100 g rat. Utilizing the same number as 30 000 glomeruli/kidney the total renal blood flow is calculated at 2.1 ml/min per 100 g rat. This is in reasonable agreement with the figures for the total renal blood flow found in the present series. As a matter of fact this ought to be expected as essentially no trapping of the spheres was found in other structures than in the glomerular capillaries.

Another source of error is that some spheres could escape trapping due to passage in shunt vessels. The finding however that the lung activity only amounted to about 1 per cent of the total amount of activity injected speaks against this possibility. The possibility that the Microfil injection could wash away the spheres trapped could also be eliminated as samples of the venous effluent during the microfilling showed nil activity.

The major findings in the present investigation are: (1) There were no differences in the blood flow and the blood flow distribution between the left kidney prepared for micropuncture and the right untouched kidney. (2) The two consecutive microsphere injections gave essentially the same result indicating that administration of about 0.5 mill of microspheres did not seriously affect the hemodynamic conditions.

As far as the  $^{133}\text{Xe}$  wash-out technique is concerned it can be concluded that this method is usable for the check up of the renal blood flow and being repeatable it will permit the titration of doses of vasoactive substances, renal nerve stimulation etc. A more precise evaluation of the blood flow in the different kidney regions during different blood flow levels by compartmental analysis turned out to be uncertain.

It is furthermore satisfying that the catheter inserted into the renal artery did not have any major influence. The preparation will therefore permit the analysis of the effect of vasoactive substances with respect to the circulatory variables as well as to the tubular function as studied with micropuncture techniques.

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## Levels of Cyclic AMP and Electrical Events during Inhibition of Contractile Activity in Vascular Smooth Muscle

By

BENGT LJUNG, OLLE ISAKSSON and BÖRJE JOHANSSON

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### Abstract

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LJUNG B, O ISAKSSON and B JOHANSSON. Levels of cyclic AMP and electrical events during inhibition of contractile activity in vascular smooth muscle. *Acta physiol scand* 1975 94: 154-166.

The possible relationship between changes in electrical and mechanical activity and in tissue levels of cyclic AMP (cAMP) during relaxation of the myogenically active rat portal vein has been elucidated in response to different inhibitory stimuli. Isoproterenol  $10^{-6}$  M caused an initial pronounced inhibition of spike discharge and contractions followed by a partial recovery over the 15 min exposure period. This inhibitory response was associated with doubling of tissue cAMP after 1 min and a less pronounced increase in later measurements (5 and 15 min). Papaverine  $10^{-4}$  M caused in contrast an inhibition of electrical and mechanical activity which developed gradually over the entire 15 min exposure period. The levels of cAMP at 1, 5 and 15 min all exceeded the corresponding values obtained with isoproterenol. The two drugs in combination gave a biphasic inhibitory response associated with a sustained large increase in the level of cAMP. These drug induced inhibitions were thus accompanied by increased levels of cAMP but the different time courses of the electromechanical responses could not be related to corresponding variations in the content of cAMP. Hyperosmolality ( $150$  mM sucrose) and mechanical vibrations (100 Hz) elicited differentiated inhibitions of smooth muscle activity without changes in tissue cAMP. The mechanical effects elicited by isoproterenol, papaverine and hyperosmolality were accompanied by approximately parallel changes in electrical spike discharge. It is concluded that increased levels of cAMP are not indispensable for vascular smooth muscle relaxation and that the magnitude of pharmacologically induced inhibitions are not always closely related to the total tissue content of the nucleotide.

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In vascular smooth muscle of the single unit type as exemplified by the rat portal vein a close correlation in time is found between the electrical spike discharge and the phasic contractile activity (Funaki and Bohr 1964). There is good evidence that the action potential is involved in triggering contraction in this type of smooth muscle and it is also generally agreed that this control of the contractile system by the electrical membrane events is exerted through variations in intracellular ionized calcium. Inhibition of spike activity is an obvious mechanism for inducing relaxation of such smooth muscles but it has been shown that their contractile activity can also be influenced by mechanisms unrelated to electrical membrane events (e.g. Evans *et al.* 1958, Johansson *et al.* 1967, Somlyo and Somlyo 1968).

A new aspect on the control of smooth muscle activity was introduced by Sutherland and Rall in 1960 when they suggested that relaxation in response to catecholamines is mediated by an elevation of cyclic adenosine 3' 5' monophosphate (cAMP). The first experimental evidence for a relation between cAMP and smooth muscle relaxation was the observation that the response of rat uterus to isoproterenol was associated with an increased level of tissue cAMP (Butcher *et al* 1965). With regard to the vascular system recent investigations have shown that various drugs which produce relaxation of different vascular smooth muscle preparations increase the tissue content of cAMP (Volicer and Hynie 1971, Triner *et al* 1972, Andersson 1972). No studies of propagating vascular smooth muscle seem to have been performed in which mechanical activity, spike discharge and level of cAMP have been related to one another in the course of induced inhibitions of activity. In order to elucidate such possible relationships the present experiments on the isolated rat portal vein were performed. Changes in electrical membrane activity, mechanical force and cAMP level were analyzed when the muscle was exposed to isoproterenol, a stimulator of adenylyl cyclase activity and papaverine, an inhibitor of the phosphodiesterase enzyme (for ref. see Bar 1974). In addition the above parameters were studied when the myogenic activity of the portal vein was inhibited by increased extracellular osmolality which reduces electrical membrane activity (see Jonsson 1970) and by mechanical vibrations which seem to interfere directly with generation of force in the contractile apparatus (Ljung and Sivertson 1975). The experimental design thus allows correlation of tissue cAMP levels to the degree of inhibition when different processes in the series of events which determine activity in the vascular muscle are interfered with.

## Methods

Isolated portal vein preparations from male rats (200–250 g) of the Sprague Dawley strain were used in these experiments. The rats were killed by a blow on the neck; the portal vein was carefully dissected, cut open longitudinally at both ends and mounted for recording by one of the following experimental procedures.

*Recording of electrical and mechanical activity* in the portal vein was performed by the sucrose gap technique as described previously (Avelsson *et al* 1967). The preparation was mounted under a passive force of about 4 mN. The hepatic end of the vessel was connected to a force-displacement transducer (Grass FT 03) for recording of isometric contractions. This active part of the vein was superfused with standard Krebs solution (composition given below). The preparation was permitted an accommodation period of at least 1 h before the actual experiment started. During this accommodation period the muscle was exposed for 5 min to phenoxybenzamine ( $10^{-6}$  M) which produces irreversible  $\alpha$  receptor blockade in the portal vein. Four different test solutions were used and these were prepared by adding (1) isoproterenol ( $10^{-6}$  M), (2) papaverine ( $10^{-6}$  M), (3) isoproterenol ( $10^{-6}$  M) and papaverine ( $10^{-6}$  M) in combination and (4) sucrose (150 mM) to the standard Krebs. The test solutions could be switched in by means of a stopcock system. Transient changes of temperature in connection with shifting of the superfusion media were prevented by draining the dead space between the respective aeration chamber and the stopcock immediately before switching. The fluid volume between the stopcock and the sucrose gap system implied some delay in the application of the test solutions; this delay has been accounted for in the illustrations below.

A total of 8 sucrose-gap experiments were performed. The order of application of the test solutions was varied but the time of exposure was always 15 min for each and recovery between exposures was 30 min or more. In the following presentation of the results, emphasis will be placed on the changes in phasic electrical and mechanical activity whereas changes in the level of the resting membrane potential will be judged with great caution since they were determined by the sucrose gap method. Small variations in super-

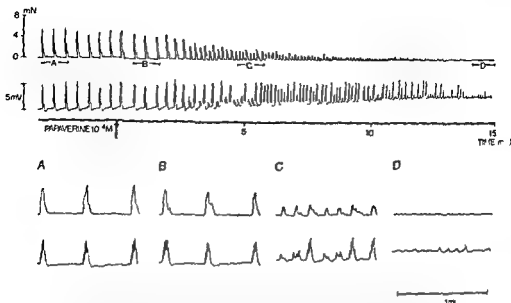


Fig. 2 Isometric contractions (upper tracing) and electrical membrane activity (lower tracing) in the rat portal vein. Upper panel illustrates control activity and changes during the 15 min following administration of papaverine ( $10^{-6}$  M) at arrow. Lower panel shows sections of same recordings on an expanded time scale.

the papaverine response is assumed. The time course of the response in Fig. 3 therefore seems to represent the superimposed time courses of the responses illustrated in Fig. 1 and 2: an immediate inhibition due to isoproterenol, a temporary recovery related to the dwindling isoproterenol effect and the delayed onset of the papaverine effect and finally the gradual decline of activity due to papaverine.

Hyperosmolality as illustrated by Fig. 4 produces an inhibitory response quite different from those caused by isoproterenol or papaverine. The frequency of burst discharges and phasic contractions decreases markedly, whereas the spike activity within the bursts and the contraction amplitude are relatively well maintained. The decrease in integrated contractile activity is most pronounced in the early phase of the response but is evident throughout the exposure period. The level of the resting membrane potential did not change consistently in the different experiments with hyperosmolality. This inconsistency was probably due to changes in flow rate of the solution as pointed out above.

The inhibitory effect on the spontaneous activity of longitudinal vibrations applied to the portal vein at a frequency of 100 Hz and  $\pm 250 \mu\text{m}$  in amplitude is illustrated in Fig. 5. For technical reasons the electrical membrane activity was not studied in these experiments. A small elevation of the line of passive force is seen during the 15 min vibration period. The contraction amplitude becomes reduced to one fourth of the control value in response to vibrations but the frequency of contractions remains unchanged. After the vibrations the spontaneous contractions regain the same amplitude as in the control situation. The imposed length changes thus reduce the isometric force developed by the smooth muscle in a stable and reversible way without affecting the temporal pattern of activity.

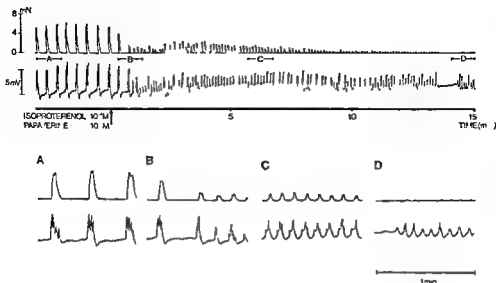


Fig 3 Isometric contractions (upper tracing) and electrical membrane activity (lower tracing) in the rat portal vein. Upper panel illustrates control activity and changes during the 15 min following administration of isoproterenol ( $10^{-6}$  M) and papaverine ( $10^{-4}$  M) in combination at arrow. Lower panel shows sections of same recordings on an expanded time scale.

The effects of the different inhibitory influences on the cAMP content and on the mechanical activity of the rat portal vein are summarized in Fig 6 and 7. The cAMP level of the smooth muscle in the control situation was determined in 8 preparations which were frozen after the spontaneous activity had been quantitated and found stable during a 20 min period. The value obtained  $10.8 \pm 0.5$  pmol/mg protein (mean  $\pm$  S.E.) is indicated as the basal cAMP level in the figures. Administration of isoproterenol  $10^{-6}$  M (Fig 6 A) decreased the mechanical activity to an average of 30 per cent of control during the first min but a gradual recovery occurred during the 15 min exposure period. The cAMP level reached a peak value during the first min ( $19.4 \pm 3.8$  pmol/mg protein) and remained elevated throughout the 15 min period of isoproterenol influence.

In contrast papaverine ( $10^{-4}$  M) caused a gradual decrease in mechanical activity so that 20 per cent remained during the first few min after administration of the drug, whereas practically all activity vanished after a 10 min exposure period. The cAMP level was already during the first min considerably increased and reached  $31.5 \pm 5.5$  pmol/mg protein in the 5 min samples.

When isoproterenol ( $10^{-6}$  M) and papaverine ( $10^{-4}$  M) were administered in combination (Fig 6 C) a biphasic inhibitory mechanical response was obtained (cf Fig 3). The concomitant increase in cAMP attained  $29.9 \pm 7.4$  pmol/mg protein during the first min of exposure to the drugs in combination; the peak value at 5 min amounted to  $45.6 \pm 8.3$  pmol/mg protein.

In summary, the increase in cAMP in response to isoproterenol was temporally correlated to the reduction in contractile activity. Papaverine caused considerable elevations in

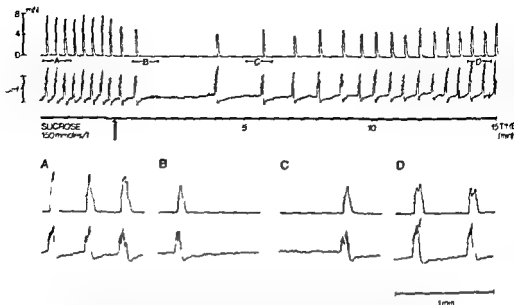


Fig. 4 Isometric contractions (upper tracing) and electrical membrane activity (lower tracing) in the rat portal vein. Upper panel illustrates control activity and changes during the 15 min following administration of hyperosmotic solution (+ 150 mM sucrose) at arrow. Lower panel shows sections of same recordings on an expanded time scale.

cAMP content within 1 min after administration of the drug, but no significant mechanical response was seen until after 3 min.

When the osmolality of the bath solution was increased by addition of sucrose 150 mM (Fig. 7 A) there was a pronounced decrease in the averaged contractile force which was sustained for the 15 min exposure period. Similarly longitudinal vibrations applied in the smooth muscle reduced the mechanical activity to 20–30 per cent of the control value during the entire 15 min period (Fig. 7 B). Neither the increased osmolality nor the induced vibrations caused any significant change in the level of cAMP.

### Discussion

The present results confirm earlier observations concerning the close temporal relation between spike discharge and phasic contractions in portal vein (Funaki and Bohr 1964, Axelsson *et al.* 1967). It is also evident from the present study that this electromechanical cor-

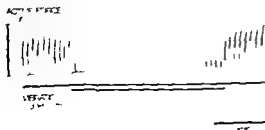


Fig. 5 Inhibitory effect of longitudinal vibrations (100 Hz,  $\pm 50$  g/m) on the spontaneous activity of the rat portal vein.

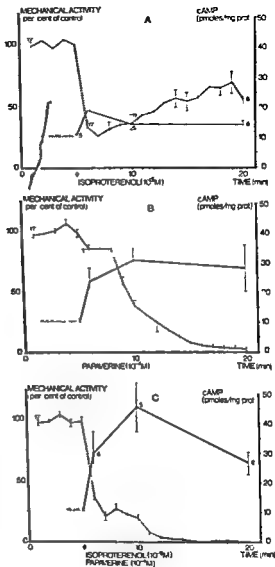


Fig. 6 Mechanical activity and cAMP levels in the rat portal vein exposed to isoproterenol  $10^{-6}$  M (A) papaverine  $10^{-4}$  M (B) or the two agents in combination (C). Mechanical activity expressed as a percentage of the averaged force developed during the 5 min control period. Shaded line indicates cAMP level in eight control preparations. Subsequent cAMP values (mean  $\pm$  S.E.) obtained from preparations frozen 5 or 15 min after administration of the drugs. Values of mechanical activity (mean  $\pm$  S.E.) obtained from all preparations studied at each time period. Number of observations indicated by figures.

relation in time persists during responses to isoproterenol, papaverine and hyperosmolality which all produce characteristic differentiated changes in the rhythmicity of the muscle. Furthermore, the size of the individual contractions during these responses seem to be related to concomitant changes in number and amplitude of spikes within the bursts although the experiments do not yield true quantitative data in this respect. The electrical membrane events thus appear intimately involved in the short term regulation of the contractile apparatus in the smooth muscle probably via rapid oscillations in intracellular  $Ca^{2+}$ . In analogy with the situation in striated muscle the phasic contraction in portal vein may be

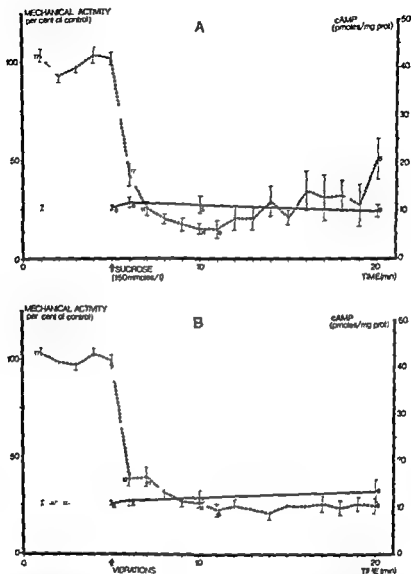


Fig. 7 Mechanical activity and cAMP levels (mean  $\pm$  SE) in the rat portal vein exposed to increased osmolality by addition of sucrose 150 mM (A) and to longitudinal vibrations 100 Hz  $\pm$  250  $\mu$ m (B). Number of observations indicated by figures.

triggered by spike activity through influx or release of calcium and relaxation brought about by sequestration of calcium in sarcoplasmic reticulum and mitochondria (Somlyo and Somlyo 1971, Andersson and Nilsson 1972).

In view of the above mentioned maintenance of electromechanical correlation under the influence of isoproterenol, papaverine and hyperosmolality it appears that these vasodilator agents exert much of their action through this short term ionic control of the contractile machinery. However, it is equally clear that the mechanical responses to isoproterenol and

papaverine are accompanied by significant elevations in the tissue content of cAMP. This is in agreement with the hypothesis of Sutherland and Rall (1960) that drug induced relaxation of smooth muscle may be dependent on increases in cAMP. The present results with hyperosmolality and mechanical vibrations illustrate on the other hand that an increased level of cAMP is not an obligatory requirement for smooth muscle relaxation in general. It seems important that the biochemical control of contractile activity which may be exerted by isoproterenol and papaverine via cAMP be reconciled with the changes induced via the short term ionic control.

Fulfillment of the following two criteria has been emphasized for the establishment of a causal relationship between a change in tissue cAMP and a physiological response. 1 changes in the tissue level of cAMP should precede the physiological response and 2 there should be a correlation between the degree of change in cAMP and the magnitude of the physiological response. We will first discuss whether the present results from the rat portal vein possibly fulfill these criteria and thus support a causal role of cAMP in this type of vascular smooth muscle.

With regard to the effect of isoproterenol the experimental protocol used in the present study does not permit a test of the first requirement due to the fact that inhibition of contractile activity was almost complete before the first measurement of cAMP was made. In the case of papaverine the increase in tissue cAMP certainly precedes the marked degrees of relaxation in that an approximately 200% rise in cAMP was already found after 1 min when only a small relaxant effect could be registered.

As to the second of the above criteria some quantitative correlation between the degree of relaxation and the tissue level of cAMP has been obtained in the present study. Thus the combination of isoproterenol and papaverine which produced the most pronounced relaxing effect was also associated with the highest tissue levels of cAMP. A comparison between the two drugs when applied individually is more difficult. Although isoproterenol caused a very rapid and pronounced relaxation the increase in the level of cAMP determined 1 min after addition was small in comparison to the increase obtained with papaverine. A typical feature of catecholamine sensitive tissues is however that stimulation of their  $\beta$ -adrenoceptors causes a biphasic increase in the level of cAMP (Dobbs and Robison 1968, Craig *et al* 1969). Therefore the peak level of cAMP in response to isoproterenol might have occurred very early before the first measurement was made in the present study. The differences in the time course of the inhibitory mechanical responses obtained with isoproterenol, papaverine and the combination of the two drugs are not readily explained by the temporal pattern of cAMP levels found in this study. In bovine coronary arteries differences in the rate of onset of relaxation obtained with isoproterenol and papaverine respectively have also been observed by Poch and Kukovetz (1972). However in rabbit colon muscle Andersson (1973) found a temporal correlation between increased cAMP and relaxation induced by papaverine. At present there is no obvious explanation for the delayed inhibition of vascular smooth muscle observed with papaverine if this inhibition is to be related to the cAMP level but there are some possibilities which might be considered. The phosphodiesterase enzymes found in crude tissue homogenates are active against different purine 3',5' mononucleotides (Beavo *et al* 1970) and it is possible therefore that the level



of cyclic guanosine 3' 5' monophosphate (cGMP) may also be affected by the phosphodiesterase inhibitor papaverine. Information is now accumulating in the literature that different contracting drugs like acetylcholine, carbachol and methacholine increase the level of cGMP in smooth muscle (Schultz *et al.* 1973). Perhaps a transient increase of cGMP in response to papaverine might have counteracted the relaxing effect exerted by this drug via cAMP. Alternatively the present results might indicate that papaverine initially increases the level of cAMP mainly in a localized compartment of the cell which does not readily affect the contractile system. It has recently been reported (Andersson 1973) that papaverine stimulates cytoplasmic phosphodiesterase activity but inhibits phosphodiesterase activity in the microsomal and mitochondrial fractions of intestinal smooth muscle.

If the present experiments are taken to indicate a causal role of cAMP in the inhibitory actions of isoproterenol and papaverine on portal vein despite the above differences in time courses, the mechanisms by which the cyclic nucleotide affects the contractile apparatus must be considered. There is no clear evidence that cAMP is directly involved in the chemo-mechanical transduction at the level of the actomyosin system. It is more likely that it operates through changes in the ionic control ( $[Ca^{2+}]$ ) or the supply of energy (ATP) to the contractile machinery. The stimulating action of cAMP on the binding of  $Ca^{2+}$  to a microsomal fraction of smooth muscles (Andersson and Nilsson 1972, Andersson 1973, Baudouin, Legros and Meyer 1973) is of interest in this connection. However, the present findings indicate that cAMP does not act exclusively by enhancing the removal of cytoplasmic  $Ca^{2+}$  but also by reducing the supply of  $Ca^{2+}$  through inhibition of the electrical membrane activity. Earlier work by Somlyo and Somlyo (1969) and by Somlyo, Haeussler and Somlyo (1970) have demonstrated effects of isoproterenol, dibutyryl cAMP and theophylline on membrane potential in vascular smooth muscle and in avian slow striated muscle. Hyperpolarization was obtained with consistency only at low  $[K^{+}]$  (1 mM) whereas relaxation at higher  $[K^{+}]$  occurred without concomitant changes in membrane potential or even in association with depolarization. Isoproterenol inhibition of uterine muscle is associated with increased cAMP and hyperpolarization inversely related to  $[K^{+}]$  (Kroeger and Marshall 1973). In the present experiments performed at a  $[K^{+}]$  of 5.9 mM the inhibitory actions of isoproterenol and papaverine always seemed to be accompanied by membrane depolarization (cf. Johansson *et al.* 1977) although the sucrose gap technique does not permit definite conclusions in that respect. It appears that the influence of these drugs on membrane potential may be mediated by an effect of the increased tissue cAMP on ion permeability or electrogenic ion transport (cf. Somlyo and Somlyo 1969, Somlyo *et al.* 1972). Such effects of cAMP could also relate to the changes in phasic electrical activity which are induced by isoproterenol and papaverine but at present the possible links between these biochemical and electrophysiological events are not known.

As pointed out in the foregoing the present findings with hyperosmolality show that reduced contractile activity associated with inhibition of electrical activity can occur without variations in cAMP. Under the influence of mechanical vibrations both the level of cAMP and the temporal pattern of muscle rhythmicity remain unchanged indicating that the effect of active force in this case may occur directly at the contractile proteins (Ljunger *et al.* 1977).

son 1975) Also with regard to the responses to isoproterenol and papaverine one must in the light of the foregoing discussion seriously consider the possibility that the rise in cAMP is merely an epiphenomenon without causal role in the inhibition of mechanical activity

The present experiments have thus illustrated that inhibition of vascular activity may occur through primary influences at different locations in smooth muscle cells In producing this inhibition different patterns of biochemical and electrophysiological alteration have been found

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## Release of Gastrin on Vagal Stimulation in the Cat

By

B UVNAS K UVNAS WALLENSTEN and G NILSSON

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### Abstract

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UVNAS B K UVNAS-WALLENSTEN and G NILSSON *Release of gastrin on vagal stimulation in the cat* Acta physiol scand 1975 94 167-176

Gastrin released by electrical vagal stimulation was measured in the portal blood of eviscerated cats. Blood flow was recorded by a drop chamber technique and the total gastrin output calculated. Basal peripheral gastrin levels averaged 65 pg/ml and basal portal levels 225 pg/ml. On unilateral vagal stimulation with frequencies above 3-4 Hz gastrin release rapidly increased, reaching a peak within 3-10 min. In spite of continued stimulation and independent of frequency the gastrin levels declined and returned to basal values after a total of 2-3 000 stimuli. A new maximal response could be induced first after a recovery period of 15-30 min. In the same cat stimulation of either the left or the right vagus released equal maximal amounts of gastrin (average 32 000 pg). The maximal gastrin output on vagal stimulation corresponds to less than 1% of the total content of antral gastrin determined with radioimmunoassay.

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The concept of vagal control of gastrin release was originally formulated by Uvnäs (1942) who observed in acute experiments on cats that not only was the gastric acid secretory response to electrical vagal stimulation abolished or considerably reduced by extirpation or cocaineization of the antrum, but also that this secretory response could be re-established by cross-circulating blood from a vagally stimulated donor animal. The validity of the Uvnäs hypothesis has been confirmed by later experiments in which indirect evidence for vagal release of gastrin was obtained in dogs following sham feeding (Pe Thien and Schofield 1959 Olbe 1963) and insulin induced hypoglycemia (Burstall and Schofield 1953) and in cats after reserpinization (Emås and Fybo 1965) and electrical vagal stimulation (Fybo 1967).

Recently the use of radioimmunoassay techniques has allowed the direct demonstration of vagally induced gastrin release in dogs on sham feeding (Nilsson *et al* 1972) electrical vagal stimulation (Lancault *et al* 1973 Becker *et al* 1974) and insulin hypoglycemia (Jaffe *et al* 1970). Many observers have reported that a release of gastrin—recorded as a rise in the plasma gastrin level—occurs in humans after administering insulin (Ganguli and Elder 1971 Hansky *et al* 1971). However, plasma gastrin concentrations alone do not adequately reflect the gastrin output in response to a certain stimulus. In the present study we have

attempted to obtain quantitative information concerning gastrin output by recording simultaneously gastric blood flow and gastrin concentrations in the gastric and/or the peripheral venous blood.

### Methods

#### *Operative procedures*

The experiments were performed on 30 cats (2 to 4 kg) fasted for 18 h and anesthetized with chloralose-urethane (50 and 100 mg/kg i.v.). The lung ventilation was kept constant by artificial respiration and bronchial accumulation of mucus was minimized by continuous suction. Blood pressure was recorded in one of the femoral arteries. Animals with a blood pressure below 100 mm Hg were excluded from the study. Rectal temperature was kept at 38–39°C by heating the animal with an electric heating pad.

The intestine below the duodenum was removed. To allow sampling of antral gastrin the portal vein was ligated close to its bifurcation (i.e. above the entrance of the antral venous blood) and a polyethylene catheter was inserted into the blind end of the superior mesenteric vein. The remaining retrograde superior mesenteric blood flow (containing blood mainly from the stomach (but also blood from the duodenum and the pancreas)) was led via the catheter to a recording drop chamber unit and then back into the cat via a cannulated femoral vein (Fig. 1). This blood will be referred to as gastric blood. In order to keep the antral pH constant a catheter was inserted into the duodenum pushed up into the antrum and fixed in position by a ligature around the pylorus. During each experiment the catheter was continuously perfused with warm (38°C) isotonic 0.15 M phosphate buffer solution (pH 7.4) at a rate of 5 ml/min. The infused solution was allowed to leave the antrum by overflow through a plastic cannula inserted into the stomach just orally to the antrum-corpus boundary. The supine position of the cat and the site of fixation of the gastric cannula ensured a complete filling of the antrum with a small overflow pressure.

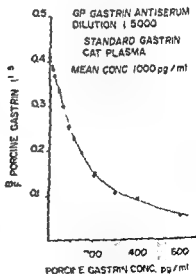
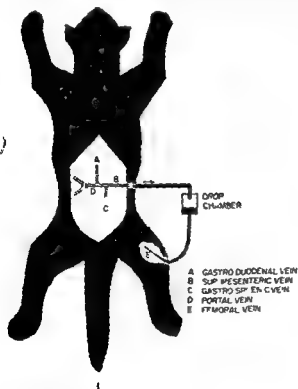


Fig. 1. Schematic drawing illustrating the technique used for recording gastric blood flow and sampling blood for gastrin determination.

Fig. 2. Ratio antibody bound (B) to free (F) <sup>125</sup>I porcine gastrin I as a function of the concentration of unlabelled porcine gastrin I and serial dilutions of cat plasma.

The cervical vagal nerves were ligated or cut and covered with paraffin oil or Plastibase (E R Squibb & Sons) to prevent drying. The distal ends of the nerves were stimulated supra maximally with a Grass stimulator (S 88) delivering stimuli of 7 V 2-5 ms duration and 1-20 Hz. The effects on heart rate and blood pressure following stimulation were taken as evidence of vagal activation.

In certain experiments gastric secretion from the gastric cannula was collected each 15 min. The volume was measured and the acidity determined by titration with 0.1 M NaOH using phenolphthalein as indicator.

#### Radioimmunoassay

Plasma gastrin concentrations were determined by radioimmunoassay using labelled porcine gastrin. Gastrin antibodies were produced in guinea pigs by injection of crude extracts from porcine antra. The assay technique permitted the detection of 0.1 pg/ml of gastrin in a standard solution. Plasma samples were assayed at dilutions of 1:10-1:100. No cat gastrin was available as standard. However when tested over a 15 fold concentration range cat plasma gastrin behaved immunochemically as the porcine gastrin standard used (Fig. 2). Further details of the radioimmunoassay have been given elsewhere (Nilsson in press).

1 ml samples of gastric and/or femoral blood were collected repeatedly during the experiments. Gastric blood was taken before it entered the drop chamber unit. Blood samples were immediately centrifuged at 2000 rpm for 10 min. The plasma was removed, frozen and kept at -70°C until the gastrin assays were performed. In order to compensate for blood loss due to the repeated sampling corresponding amounts of physiological saline were given intravenously. After completion of all surgical procedures the cats were heparinized to prevent blood clotting in the catheters. Care was taken to ensure that the heparin concentration did not exceed 10 i.u./ml.

The continuous recording of gastric blood flow allowed quantitative calculations of the gastrin output to be made. Hematocrit values for blood from 10 cats varied between 35-56—average 47.5. However measurements of the hematocrit during the relevant periods in 10 expts revealed a variation in one and the same animal of less than 10 per cent from the average value. In the present study the total gastrin output has been calculated as 65% of the product of gastric blood flow times ( $\times$ ) gastrin plasma concentration. The basal gastrin output/min was calculated from 3-5 gastric blood samples taken during 10 min and the concomitant gastric blood flow. To obtain the gastrin release due to vagal stimulation the basal gastrin output was subtracted from the total amount of gastrin released into the gastric vein.

## Results

### Basal output of gastrin

(a) *Peripheral venous blood* The basal gastrin levels in the peripheral blood varied considerably between cats—in 15 cats between 34 and 111 pg/ml (mean 65 pg/ml) (Table I). How

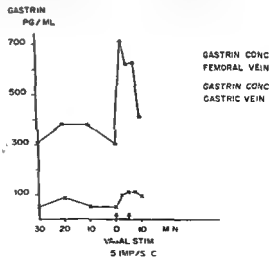


Fig. 3 Gastrin in samples of gastric and peripheral venous blood taken simultaneously. The effect of vagal stimulation 5 Hz for 5 min. Note the 5 fold difference in basal gastrin concentration between gastric and peripheral blood and the abrupt rise in gastrin level in gastric blood. The accompanying rise in the peripheral venous blood is reduced due to the repeated sampling of gastrin rich gastric blood.

TABLE I Basal plasma levels of gastrin in (a) peripheral and (b) gastric venous blood. Individual and mean values in 15 cats.

Cat no	Sample no					Mean value
	1	2	3	4	5	
(a) Mean value from all cats ■ pg/ml (34-111)						
1	160	85	75	125		111
2	10	50	40			40
3	60	75	60			65
4	80	80	75	60		74
5	110	110	110	95		106
6	50	75	50	50		56
7	50	40	50			47
8	55	45	40	45	45	46
9	43	55	55	55		57
10	50	45	40	45		45
11	37	33	33	33		34
12	50	40	55	40	50	45
13	95	95	75	80	100	89
14	100	100	117	100	100	10
15	60	65	60			67
(b) Mean value from all cats 224 pg/ml (65-605)						
1	580	600	600	600		605
2	150	90	150	150		135
3	180	170	180			177
4	100	75	100	110		96
5	150	150	100			167
6	65	90	90			8
7	100	190	100	00	210	110
8	35	275	350			317
9	140	100	140	160		135
10	180	310	110	300	190	38
11	75	45	75			64
12	80	80	110	80		90
13	100	100	100			00
14	310	380	380	310		345
15	400	550	550	550		517

ever within experiments gastrin concentrations fluctuated less and were usually within 30% of the mean value.

(b) *Gastric venous blood* In general the gastrin concentration in the gastric outflow was found to be about 4-5 times higher than in the peripheral blood (Fig. 3). The average gastrin values from 15 cats varied between 65 and 605 pg/ml—mean value 224 pg/ml (Table II). Provided that the gastric blood flow remained constant the basal gastrin levels in the gastric blood showed variations of the same magnitude as in the peripheral blood during the course of an experiment. When increases in the basal gastrin concentrations were observed they were usually found to be due to a decrease in gastric blood flow and not to an increase in gastrin output. Calculations showed the gastrin output to be unchanged.

#### Vagally induced release of gastrin

*Significance of stimulation frequency* Adequate vagal stimulation was able to induce an abrupt increase in gastrin release reflected as a rise in the gastrin level not only in the gastric

TABLE II Gastrin release into the gastric vein following unilateral vagal stimulation on 8 cats with  $> 2400$  impulses

Cat no	Frequency Hz	Duration sec	Number of impulses	Gastrin pg
1	10	470	4,400	11,525
2	4	600	2,400	8,400
	4	600	2,400	7,600
3	4	900	3,600	52,500
	8	900	7,200	47,213
	11	600	9,000	45,575
4	8	300	2,400	34,188
	11	300	2,400	33,463
5	5	480	2,400	14,850
6	10	300	3,000	32,950
7	8	300	2,400	58,000
8	5	900	4,500	41,200

Mean value from 8 experiments  $37,000$  pg ( $7,600$ – $58,000$ )

(Figs 3 and 4) but also in the peripheral venous blood (Fig 3). The stimulation frequency was critical. On unilateral stimulation with frequencies below  $2$ – $3$  Hz no significant rise in the gastrin levels (Fig 4) was observed in the gastric blood. Attempts to establish a frequency response curve for the gastrin release met with unexpected difficulties since the release mechanism proved to be rapidly fatigued. Within the frequency range studied ( $4$ – $20$  Hz) vagal stimulation induced a rise in the gastrin concentration reaching a peak level

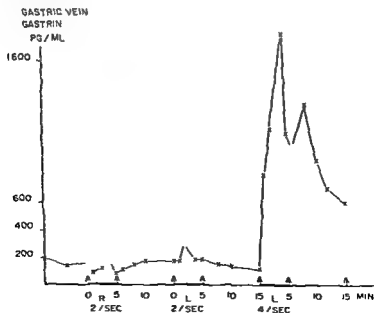


Fig 4 Release of gastrin on unilateral vagal stimulation ( $4$  Hz  $5$  min). Note that there is no significant release with  $1$  Hz. R=right, L=left vagal nerve



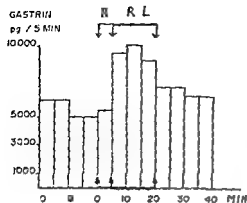


Fig. 5 Gastrin release on unilateral and bilateral vagal stimulation with 2 Hz. Note the absence of response to unilateral stimulation R = right L = left

within 5-10 min. Within 10-20 min the gastrin values then declined to near prestimulatory levels regardless of continued stimulation (Figs 6 and 7). The higher the frequency the steeper and higher the gastrin peak and the shorter the release response provided that the blood flow was unchanged.

Further analysis of several experiments exhibiting the fatigue phenomenon revealed that within a frequency range of 4-20 Hz (unilat. stim.) the gastrin release mechanism became temporarily exhausted when the total number of stimuli delivered exceeded 2-3 000. This

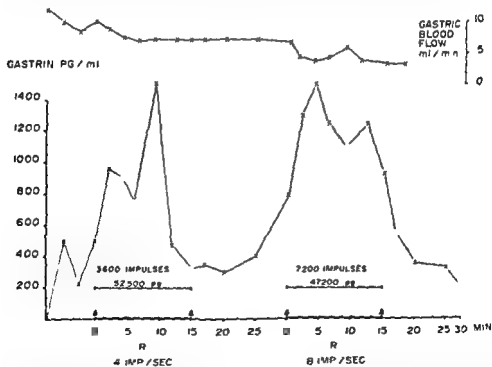


Fig. 6 Release of gastrin following vagal stimulation (4 and 8 Hz for 15 min) illustrating fatigue of the release mechanism and subsequent recovery after 15 min. Note the decreased gastric blood flow during the second stimulation giving higher gastrin plasma concentrations but unchanged total output. R = right

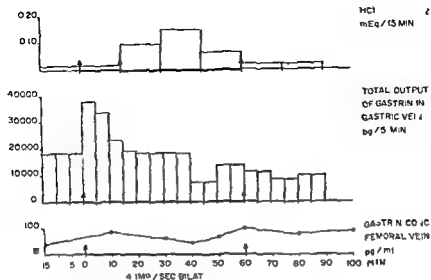


Fig 7 Gastrin output into the gastric vein, gastrin concentration in the femoral vein and acid secretory response following bilateral vagal stimulation (4 Hz 60 min). Note the continued gastric acid production in spite of declining gastrin release into the gastric vein.

was evidenced by a return of vagally induced gastrin levels to basal as soon as 2-3 000 impulses had been delivered in spite of continued stimulation (Fig 6). The fatigue of the release mechanism occurred independently of frequency or length of stimulation as soon as this critical number of impulses was reached. As is evident from Table II, the total output of gastrin in response to a supramaximal stimulation varied considerably from animal to animal (7 800-58 000 pg). However, within one and the same animal the maximal amounts of releasable gastrin were fairly constant (Table II, Fig 6). Stimulations with more than 2-3 000 impulses did not augment the gastrin response (Table II). After depletion 15-20 min were required for full recovery.

Stimulation of either the right or the left cervical vagus released gastrin (Figs 4 and 6). Bilateral stimulation increased the total gastrin output and caused a significant rise in the gastric blood with stimulation frequencies as low as 2 Hz (Fig 5).

#### *Importance of concomitant recording of gastric blood flow and gastrin concentration*

Initial gastric blood flow varied between 4 and 10 ml/min. A slight decline was sometimes seen during the course of the experiments. Fig 6 illustrates the importance of simultaneous recording of gastric blood flow and plasma gastrin concentrations for obtaining correct quantitative information about gastrin release. In this experiment, vagal stimulation with a supramaximal impulse load (3 600 impulses) induced the release of 52 500 pg of gastrin. A second vagal stimulation, this time with twice as many impulses—7 200—resulted in a more sustained release of gastrin. From the concentrations of gastrin one might get the impression that the second gastrin release response (7 200 impulses) was higher than

the first one (3 600 impulses). However, since for unknown reasons the gastric blood flow declined during the second stimulation the total gastrin output was in fact lower (47 200 pg compared to 52 500 pg).

Because an antrum buffer perfusion system was used in our studies gastric acid was not collected and measured routinely. To find out whether the surgical preparation might have deranged the acid secretory response pattern to vagal stimulation gastric acid was collected and measured in some experiments. In these experiments the animal was placed on its left side in such a way that the stomach was continuously drained via a plastic catheter in the gastric cannula. As illustrated in Fig. 7 vagal activation induced not only the expected temporary rise in the gastrin level in the gastric blood but also a continuous acid secretory response typical of vagal stimulation. The quantitative relationship between gastrin output and the acid secretory response will be discussed in a subsequent paper.

### Discussion

The present results corroborate the 30-year old proposal of Uvnäs (1942) concerning vagal control of the release of gastrin in cats. Previously Lanciault *et al.* (1973) and Becker *et al.* (1973) reported increased gastrin levels in the portal and antral veins respectively on electrical vagal stimulation in dogs. Although there is principal agreement between the present results on cats and those previously reported on dogs there are at the same time striking quantitative differences which require some comments.

Since cat gastrin was not available the gastrin values obtained with our immunoassay are only relative. However, our cat plasma gastrin has been shown to behave immunochemically in the same way as the porcine gastrin standard. Furthermore the mean peripheral plasma value found in our cats (65 pg/ml) is of the same magnitude as found in other species (e.g. man and dog). These facts indicate that our immunoassay technique is reliable.

We observed a 4–5 fold higher basal gastrin concentration in the gastric blood than in the peripheral venous blood. The gastrin concentration in the gastric blood increased on vagal stimulation up to 10-fold reaching a peak after only a few minutes. The higher the frequency of stimulation and the more rapid the gastric blood flow the quicker the peak of gastrin output appeared. At a frequency of 20 Hz, as used by Becker *et al.* for example (see below) we observed a peak output already within 30 s.

In spite of continued stimulation the gastrin release rapidly fell to reach the prestimulatory level within 10–20 min. Such a fatigability was observed after stimulation with a total of 2 000–3 000 impulses provided the stimulation frequency was in the range of 4–20 Hz. Full recovery of the release mechanism required a resting period of 15–20 min.

In contrast to our results Lanciault *et al.* found in their dogs no difference between the basal gastrin levels in the portal and the peripheral venous blood. Further they reported a 2–3 fold increase in portal gastrin levels even after 30 min continuous stimulation of the cervical vagi (frequency 5 Hz).

The discrepancies between our results and those of Lanciault *et al.* may be due to technical factors. They determined gastrin concentrations in portal blood withdrawn from a

catheter inserted into the portal vein. No control of the position of the tip of the catheter is mentioned and with such a sampling technique an uncontrollable admixture of blood from non antral sources cannot be avoided. In fact their finding of almost equal portal and peripheral prestimulatory gastrin levels indicates that in their dogs antral blood formed only a minor part of the portal blood withdrawn. In the absence of simultaneous blood flow measurements their report of a 2-3 fold elevation of the portal gastrin level even after 30 min continuous vagal stimulation is of questionable validity. The elevation might equally well be explained as being due to reduced blood flow or less admixture of non antral blood as to vagally induced gastrin release.

The pronounced fatiguability of the gastrin release mechanism to vagal stimulation observed in our study may have several explanations. A rapidly occurring fatigue of vagal neurotransmission processes cannot be excluded but seems rather unlikely especially in view of the recovery time (15-20 min) which is unreasonably long for cholinergic transmission fatigue. In our view our observations indicate the existence of a small antral pool of gastrin easily depletable by vagal impulses. Similarly in the dog the gastrin release mechanism seems to be easily exhausted. Electrical vagal stimulation also in this species leads to a transient rise in gastrin output (unpublished observation).

The reason why the existence of this depletable gastrin pool has been overlooked by previous investigators is probably the different sampling techniques used. In order to detect and measure quantitatively the transient initial gastrin peak repeated blood sampling is required during the first few minutes of stimulation. In the experiments of Lanciault *et al* the initial gastrin peak has almost certainly been missed since the first blood sample was not taken until after 30 min of stimulation.

The average size of the depletable gastrin pool was 32 000 pg (see Table II) indicating that only a minor part of the total antral gastrin is immediately available for release by vagal impulses. Nilsson *et al* (1973) have determined the total content of gastrin like material in cat antrum to be about  $10^7$  pg. Under the assumption that the immunoreactive material corresponds to gastrin or closely related precursor material less than 1% of the total gastrin store should be released by one supramaximal vagal stimulation (2 000-3 000 impulses).

The stimulation frequency used by us to demonstrate the fatiguability of the gastrin release mechanism ( $>4$  Hz) is probably unphysiologically high. The physiological vagal impulse frequency range is assumed to be around 0.5-4 Hz as graded acid responses are obtained following vagal stimulation within this frequency range (Sjodin 1975). Also stimulation with such low frequencies suffices to induce and maintain HCl secretion for hours—suggesting a concomitant release of gastrin. We did not regularly record the gastric secretory response to vagal stimulation but in those experiments in which the HCl secretion was measured a sustained secretion was observed throughout the experiments as illustrated in Fig. 7.

In our experiments unilateral vagal stimulation with frequencies below 4 Hz did only rarely cause a detectable rise in the gastrin output. This inability to record any gastrin release at frequencies within the physiological frequency range are most certainly due to deficiencies in our recording technique. As already mentioned under Methods, our gastric blood contains a mixture of venous blood mainly from the antrum and the corpus of the

stomach (but also from the pancreas and the duodenum) the blood from the antrum forming a minor part. Consequently the original gastrin concentrations in the antral blood will be considerably reduced due to the admixture with blood relatively poor in gastrin. Since the basal gastrin values fluctuate about 30%, our present technique will not always allow us to record the low gastrin output which can be expected on unilateral vagal stimulation with frequencies below 4 Hz. Bilateral stimulation increased the gastrin output compared to unilateral stimulation. Thus bilateral stimulation with 2 Hz raised gastrin output appreciably (Fig. 5).

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## Reflex Inhibition of the Slowly Adapting Stretch Receptors in the Intact Abdomen of the Crayfish

By

A NÅL and L WALLOE

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### Abstract

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NÅL A and L WALLOE. *Reflex inhibition of the slowly adapting stretch receptors in the intact abdomen of the crayfish* Acta physiol scand 1975 94 177-183

The reflex inhibition of abdominal stretch receptors in crayfish was studied by controlled passive flexion of individual abdominal joints. The results are very similar to those obtained by electrical or mechanical stimulation of individual stretch receptors in immobilized abdomens. Reflex effects from posterior to anterior abdominal segments are somewhat stronger than in the opposite direction. Flexion of one abdominal joint excites the stretch receptors of that joint and inhibits the stretch receptors in neighbouring abdominal segments. Without reflex inhibition flexion of one abdominal joint excites the stretch receptors in several abdominal segments.

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The slowly adapting stretch receptors in the thorax and abdomen of the crayfish have provided unusual opportunities for the investigation of sensory inhibition. The histology of the receptors and their efferent innervation has been described in lobster and crayfish (Alexandrowicz 1951, 1952, 1967; Florey and Florey 1955). The peripheral cell body attached to a specialized muscle strand, facilitates isolation of functioning receptors and allows adequate activation by stretch applied to the muscle strand, application of various solutions, stimulation of inhibitory axons, and recording of synaptic potentials and injection of currents with an intracellular microelectrode. These technical advantages have made possible detailed studies on the inhibitory synaptic mechanisms and transmitter action of the large accessory neuron which is the main inhibitory axon (Kuffler and Eyzaguirre 1955; Hagiwara, Kusano and Saito 1960).

There is one slowly adapting stretch receptor and one large accessory neuron on each side of each abdominal segment. When the neural connections between the receptor and the central nervous system are intact, activity in one slowly adapting stretch receptor reflexly activates its own large accessory neuron. In addition, this receptor activates the large accessory neurons of several other abdominal segments, mainly on the same side of the animal (Eckert 1961). The distribution of this large accessory reflex to different abdominal segments and the input-output relations of the various reflex connections have been determined by electrical and mechanical stimulation of receptors in immobilized abdomens.

(Eckert 1961 Jansen Njå and Walloe 1970 E Jansen Njå Ormstad and Walloe 1971 a b) Page and Sokolove (1972) recently published a report which emphasized the non symmetrical distribution of the reflex. They found considerably stronger reflex connection from posterior to anterior segments than in the opposite direction when receptors were activated by spontaneous or command fibre induced abdominal flexions. The asymmetry of the reflex coupling is small during electrical stimulation of the stretch receptor axons (Jansen *et al* 1970 b).

Because of this discrepancy we investigated the input-output relations of the large accessory reflex during controlled passive flexion of individual abdominal joints. The pattern of stretch receptor activation thus obtained has several distinctive features: (1) Simultaneous and equally strong activation of stretch receptors on both sides of the animal. (2) Selective activation of slowly adapting stretch receptors because the rapidly adapting receptors respond only to full range flexions of high velocity (Njå and Walloe 1973). (3) Possible activation of other kinds of receptors with reflex connections to the large accessory neurons. To understand the *in situ* performance of the large accessory reflex results which have been obtained by electrical and mechanical stimulation of the receptors of immobilized abdomens should be supplemented by experiments in which the receptors are activated by joint flexion. In this report we show that the reflex activation produced by passive joint flexion in intact abdomens is very similar to that produced by direct electrical or mechanical stimulation of the receptors.

### Methods

The experiments were performed on the isolated abdomen of fresh water crayfish (*Asiaticus fl. rivulalis*). The preparations were bathed in oxygenated saline containing (mM): NaCl 205, KCl 5.4, CaCl<sub>2</sub> 13.5, MgCl<sub>2</sub> 2.6, Tris 10, titrated to pH 7.2 to 7.4 with maleic acid. The temperature of the bath was thermostatically controlled at 12°C. The stretch receptors were activated by controlled passive flexion of the abdominal joints (Fig. 1). Most of the experimental set up and the technique for flexing the joints have been described earlier (Njå and Walloe 1973).

Action potentials were recorded extracellularly with flexible suction electrodes fitted into circular boreholes (Fig. 1). No suction was needed since the rubber coated electrode shaft stuck to the borehole after it had been inserted as far as necessary to obtain good contact between electrode and nerve branch of the second root (inner along the extensor muscles). Situated close to the joint axis, the electrode-to-nerve contact was unaffected during the full range of movements in the joint. The amplifier activity was displayed on a cathode ray tube and photographed conventionally on moving film. Frequencies were counted over 1 s starting 0.5 s after the beginning of the step.

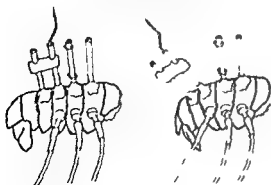


Fig. 1. Typical experimental arrangement. 3 simultaneous recordings are made from the right side of the second, third and fourth segments. Left: Zero reference position. Right: Selective flexion of the joint between the third and the fourth segments.

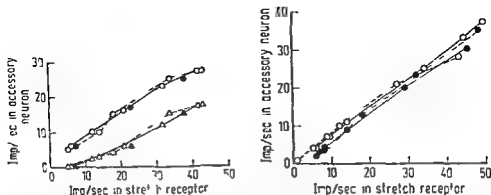


Fig. 2. Reflex output from the large accessory neuron as a function of slowly adapting stretch receptor input. The stretch receptor on the right side of the third segment was activated by pulling the receptor muscle with a fine glass hook (filled symbols) in between 2 series of flexion experiments (open symbols). Curves represent the reflex output in the same segment and transmits the reflex output in the next anterior segment. Contralateral stretch receptors were denervated. Solid lines: First series of flexion experiments. Dashed lines: Second series of flexion experiments.

Fig. 3. Reflex output from the large accessory neuron as a function of bilateral (○) and ipsilateral (●) slowly adapting stretch receptor input. The recording was made from the right side of the second segment during flexion. Ipsilateral input was produced by denervation of the receptor on the opposite side. Two trials (solid and dashed lines) were performed in each situation.

In the experiments in which the reflex effects of pulling the receptor muscle were compared to those elicited by joint flexion, the dorsal part of the tergite of abdominal segments 2, 3 and 4 were removed to expose the stretch receptors laterally. The stretch receptors on the left side of these segments were denervated. The positions of the joints were controlled by means of pins inserted vertically through the animal on the left side, one in each segment from no. 2 through no. 5 instead of glass rods in the midline.

## Results

### *Input-output relations of the large accessory reflex*

Impulse activity in a slowly adapting stretch receptor caused reflex activation of its own large accessory neuron. In addition, the large accessory neurons of several other abdominal segments were activated. Receptor activation by joint flexion produced the same reflex output as that produced by direct stretch of the receptor muscle strand, provided that the contralateral stretch receptor had been denervated.

The results from one experiment are illustrated in Fig. 2, which shows the ipsilateral reflex outputs from the large accessory neurons in the same and neighbouring segments as a function of impulse frequency in the slowly adapting stretch receptor. There was no difference in the reflex output produced either by flexion or by direct mechanical activation of the receptor. 5 experiments on 4 animals all showed similar results.

The effect of bilateral stretch receptor input was compared to that of unilateral input in several experiments. Ipsilateral input was obtained after cutting the nerve on the opposite side. Fig. 3 shows the results from a typical experiment. No difference occurred for most input frequencies. For input frequencies below about 15 imp/s, however, bilateral input was more effective than ipsilateral input.



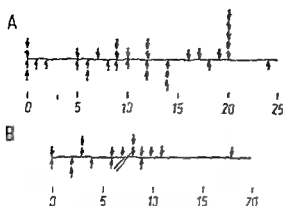


Fig. 4. Efficiency of the large accessory reflex couplings in the anterior and the posterior directions. Data from 17 pairs of large accessory reflexes between neighbouring segments. The input frequency was 20 imp/sec. A: The arrows represent the output frequencies in imp/sec for the reflexes in the anterior (above the line) and the posterior (below the line) directions. B: Similar representation of the differences within pairs (anterior direction - posterior direction). Positive differences are shown above the line.

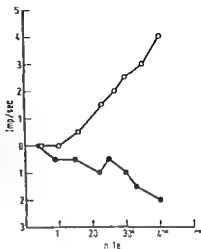
The distribution of this reflex to different abdominal segments is of obvious functional significance. Page and Sokolove (1972) recently published a report that emphasized the non-symmetrical distribution of the reflex. They found considerably stronger reflex connections from posterior to anterior segments than in the opposite direction and suggested that this could have important consequences in reflex function. We studied the symmetry of reflex coupling in 17 pairs of ipsilateral reflexes between neighbouring segments. In Fig. 4 A the reflex output (in imp/s) evoked by a stretch receptor input frequency of 20 imp/s is shown for each of the 34 reflexes. The difference between the output frequencies in the two directions appears to be small. The point estimate of the median of the output frequencies is 12 imp/s in the anterior direction and 9 imp/s in the posterior direction. The corresponding confidence intervals with a confidence coefficient of 0.95 are 9 imp/s  $< \eta < 20$  imp/s and 5 imp/s  $< \eta < 14$  imp/s (non-parametric estimates see Hoether 1971). Fig. 4 B shows the difference in output frequencies within each particular pair of reflexes. In most pairs the output frequency in the anterior direction is greater than that in the posterior direction. The difference, however, is not significant at the 5 per cent level in the present material ( $\alpha = 0.05$  in the Wilcoxon's test for paired comparisons, two-sided test).

#### Effects of flexing neighbouring joints

Reciprocal inhibition between slowly adapting stretch receptors of neighbouring segments has previously been demonstrated during electrical and mechanical stimulation of the receptors in immobilized abdomens (Léclerc 1961; Jansen *et al.* 1970a). This mutual inhibitory interaction was also observed during joint flexion in the present series of experiments.

An example is shown in Fig. 5. A slowly adapting stretch receptor was firing at about 5 imp/s after adaption to a constant angle of flexion in its joint. The change in firing frequency in this receptor caused by flexing the next posterior joint was then recorded. Flexing this joint caused a decrease in the firing frequency of the receptor. The inhibitory effect increased with increasing angles of flexion in the next posterior joint and at 40° the receptor almost stopped firing. Fig. 5 also shows the results obtained when the experiment was repeated on the same receptor after its nerve supply had been cut. In this case flexion of the next posterior joint caused a mechanical excitation of the receptor.

Fig 5 Effect of flexing a neighbouring joint. The change in firing frequency of a slowly adapting stretch receptor in the third segment is plotted as a function of the angle of flexion in the next posterior joint with intact central connections (●) and after nerve section (○). The background firing frequency of the receptor in the third segment was approximately 2 imp/s after adaption to a constant angle of flexion (30°) in its joint.



The actual effect of the neural inhibition corresponds to the difference between the two curves in Fig 5. This difference is considerably greater than the net decrease in impulse frequency evoked by joint flexion (compare with Fig 2 in Jansen et al 1970a). Flexion of the next anterior joint produced similar results.

The excitatory effect of neighbouring joint flexion on slowly adapting stretch receptors was regularly observed after nerve section in these preparations. Because it occurred after the receptors had been denervated this excitation appears to be purely mechanical. The degree of excitation was different in different preparations but it was usually stronger in the anterior than in the posterior direction. A typical example is shown in Fig 6. Maximal flexion of one joint evoked a firing frequency of about 80 imp/s in its own stretch receptors (without reflex inhibition) and increased the impulse frequency in the receptors of the next anterior segment by about 8 imp/s. The excitatory effect was about half this great in the opposite direction. Similar but weaker excitatory effects were observed 2 segments away. Provided that the steady state background firing frequency in the receptor was above zero the results were largely independent of the value of the constant angle of flexion in its joint.

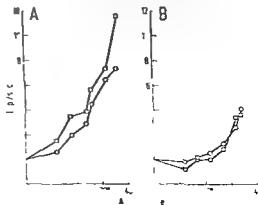


Fig 6 Effect of flexing the neighbouring joints. The change in firing frequencies of the denervated right (●) and left (○) slowly adapting stretch receptors of the third segment is plotted as a function of the angle of flexion in the next posterior (A) and the next anterior (B) joint. In this preparation, initial flexion of the next anterior joint released the receptors slightly. The background firing frequencies of the receptors of the third segment were approximately 5 imp/s after adaptation to a constant angle of flexion (30°) in their joint.

## Plasma Renin Activity and *in vitro* Synthesis of Aldosterone by the Adrenal Glands of Rats with Spontaneous, Renal, or Pinealectomy-Induced Hypertension

By

HEIKKI KARPPANEN, SALME LAHOVAARA, PERKA MANNISTO and HEIKKI VAPAATALO

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### Abstract

KARPPANEN H, S LAHOVAARA, P MANNISTO and H VAPAATALO. Plasma renin activity and *in vitro* synthesis of aldosterone by the adrenal glands of rats with spontaneous, renal or pinealectomy induced hypertension. Acta physiol scand 1975 94 184-188

In the present study a comparison was made on the role of the renin-aldosterone system in rats with various forms of experimental hypertension (pinealectomy induced, renal and spontaneous). The plasma sodium and potassium concentrations as well as renin activity were measured. The *in vitro* production of aldosterone by quartered adrenal glands of these rats was also determined. 5 weeks after the operations the blood pressure of the pinealectomized and renal operated rats was significantly increased. The plasma sodium concentration did not differ in various groups, but that of potassium was decreased in the renal hypertensive animals. The plasma renin activity of the pinealectomized rats was elevated while in other forms of hypertension it was at the control level. The basal aldosterone production by the adrenal quarters was equal in all the groups. ACTH, dibutyryl cyclic adenosine 3',5' monophosphate (DBA) and SHH stimulated the aldosterone production. The responses to ACTH and DBA were greater in the adrenals of renal hypertensive rats than in the other forms of hypertension or in the controls. We suggest that the renin-aldosterone system is of importance in the maintenance of renal hypertension while in pinealectomy induced hypertension elevated plasma renin activity reflects an increased sympathetic activity which probably is the main cause of hypertension in these animals.

**Key words.** Experimental hypertension, spontaneous hypertension, renal hypertension, pinealectomy induced hypertension, renin-aldosterone system.

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In order to elucidate the etiology and pathogenesis of human arterial hypertension various animal models have been utilized. Animals rendered hypertensive by operations affecting the renal arteries or the renal tissue itself have been used for studies of renal hypertension. Okamoto and Aoki (1963) introduced a strain of spontaneously hypertensive rats which have been generally accepted as the best model available for studies of human essential hypertension. Recently we have reported that rats with pinealectomy induced hypertension bear some similarity to the spontaneously hypertensive rats (Karppanen 1974).

Despite extensive studies there is no agreement about the significance of the renin-angiotensin-aldosterone system in the development and maintenance of arterial hypertension. In the present work we have compared the renin-aldosterone mechanism in rats with renal spontaneous or pinealectomy induced hypertension by measuring the plasma renin activity and the *in vitro* production of aldosterone by the adrenal glands of these rats.

### Materials and Methods

Male Wistar rats 170-180 g. were pinealectomized with the stereotaxic electrocoagulation method previously described in detail by us (Karppanen *et al.* 1970; Karppanen 1974). The renal hypertension was induced by removing the right kidney and tightening a figure-of-eight ligature around the remaining kidney. The spontaneously hypertensive rats were males of the Wistar strain developed by Okamoto and Aoki (1963). Unoperated normotensive male Wistar rats served as controls in the experiments. All rats received standard rat pellets (Hankkija Oy, Helsinki) and tap water *ad libitum*. The systolic blood pressure of unanesthetized rats was measured with the tail cuff method of Friedman and Freed (1949). The details of the methodology have been described previously by us (Karppanen *et al.* 1973a).

Five weeks after the operative procedures the rats were sacrificed by decapitation. Blood was collected for the determination of sodium and potassium by flame photometry and for the assay of plasma renin activity with the biological method of Boucher *et al.* (1964). The adrenal glands were quickly removed into ice-cold Krebs-Ringer bicarbonate buffer prepared free from fat and quartered with a razor blade. The details of the incubation of adrenal quarters as well as the measurement of aldosterone with a chromatographic method have been described previously by Erbiler (1971). The following drugs were used: ACTH (Acethropan® Farberwerke Hoechst AG, Frankfurt/Hoechst), N<sup>6</sup>,2'-O-dibutyryl cyclic adenosine 3',5'-monophosphate (DBA, Boehringer Mannheim GmbH, Mannheim/Waldhof), 5-hydroxytryptamine creatinine sulphate (Fluka AG, Buchs).

Student's *t*-test was used in the statistical analysis of the results.

### Results

#### Blood pressure (Fig. 1)

Five weeks after the operation when the adrenal glands were taken for *in vitro* experiments the blood pressure of the pinealectomized rats was on the average 16 mmHg higher than the blood pressure of the control rats ( $p < 0.01$ ). The blood pressure of those rats which had undergone the renal operation was 35 mmHg higher ( $p < 0.001$ ) and that of the spontaneously hypertensive rats 57 mmHg higher ( $p < 0.001$ ) than in the unoperated control rats.

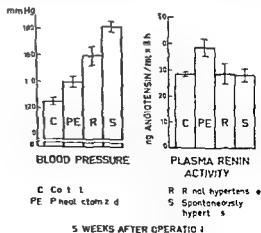


Fig. 1. Blood pressure and plasma renin activity in rats with pinealectomy induced renal or spontaneous hypertension. The values were measured 5 weeks after pinealectomy or the removal of the right kidney and tightening a figure-of-eight ligature around the left kidney.

TABLE I Effect of ACTH DBA and 5HT on the release of aldosterone from rat adrenal quarters in vitro

Stimulant	Control	PE	RH	SH
None	0.47±0.02	0.38±0.06	0.51±0.09	0.46±0.07
ACTH 5 × 10 <sup>-7</sup> M	1.21±0.07	1.14±0.15	1.45±0.18	1.04±0.08 <sup>a</sup>
DBA 5 × 10 <sup>-6</sup> M	1.7±0.15	0.84±0.05 <sup>c</sup>	1.48±0.06	0.76±0.16 <sup>b</sup>
5HT 1 × 10 <sup>-6</sup> M	0.89±0.05	0.85±0.04	0.85±0.04	1.03±0.14

The values indicate the release of aldosterone in µg per mg N during the 2 h incubation. Means ± S.E. of 4-5 incubations are given.

PE = pinealectomized RH = renal hypertensive SH = spontaneously hypertensive rats

<sup>a</sup> p < 0.05 vs renal hypertensive rats

<sup>b</sup> p < 0.01 vs renal hypertensive rats

<sup>c</sup> p < 0.001 vs renal hypertensive rats

### Plasma renin activity (Fig. 1)

5 weeks after the operation the plasma renin activity of the pinealectomized rats was increased by 35% (p < 0.01). In the rats with renal or spontaneous hypertension the plasma renin activity did not differ from control values.

### Production of aldosterone (Table I)

The basal release of aldosterone from the adrenal quarters was similar in all groups. ACTH (5 × 10<sup>-7</sup> M), DBA (5 × 10<sup>-6</sup> M) as well as 5HT (1 × 10<sup>-6</sup> M) significantly increased the release of aldosterone (p < 0.05 to < 0.001). The response to ACTH and DBA was greater in the adrenal glands of the rats with renal hypertension than in those of the spontaneously hypertensive or pinealectomized rats. The response to 5HT was similar in all groups.

### Plasma electrolytes (Table II)

The plasma sodium concentration was similar in all groups. In the rats with renal hypertension the plasma potassium was decreased (p < 0.001) as compared to the unoperated control rats. In the pinealectomized and spontaneously hypertensive rats the plasma potassium concentration was unchanged.

TABLE II Plasma sodium and potassium in rats with pinealectomy induced renal or spontaneous hypertension

Rat group	Plasma sodium mEq/l (mean ± S.E.)	Plasma potassium mEq/l (mean ± S.E.)	n
Control	137 ± 1	5.1 ± 0.1	14
Pinealectomized	139 ± 1	5.2 ± 0.1	10
Renal hypertensive	133 ± 1	4.6 ± 0.1	10
Spontaneously hypertensive	139 ± 1	4.9 ± 0.1	8

The electrolytes were determined 5 weeks after pinealectomy or the removal of the right kidney and ligation of a figure of eight ligature around the left kidney.

p < 0.001 vs unoperated control rats

## Discussion

Several investigators have suggested that the renin aldosterone system may become relatively unimportant in the maintenance of chronic renovascular hypertension in animals with one kidney removed (Vapaatalo *et al* 1970 Brunner *et al* 1971 Krieger *et al* 1971 Pals *et al* 1971 Brunner *et al* 1972). In agreement with this in the present study the plasma renin activity of rats with renal hypertension was at the level of the controls 5 weeks after the constriction of one kidney and contralateral nephrectomy. However in dogs previously subjected to unilateral nephrectomy both plasma renin activity and arterial blood pressure rise within a few minutes after renal artery constriction (Gutmann *et al* 1972). Miller *et al* (1972) demonstrated that the increased plasma renin activity is responsible for the initiation of the elevated blood pressure in such dogs. Experiments with specific angiotensin antagonists indicate that the renin angiotensin mechanism accounts for the elevation of blood pressure in the acute phase of renal hypertension also in rats (Pals and Fulton 1973). In the present study the adrenal glands of the rats with chronic renal hypertension showed an increased synthesis of aldosterone in response to stimulation with ACTH or DBA. This may further point to the importance of the renin aldosterone mechanism in the renal hypertension. In agreement with our previous works (Karppanen *et al* 1970 Karppanen *et al* 1973 ■ Karppanen 1974) the plasma renin activity of the pinealectomized rats was increased. In the present work the plasma renin activity of the spontaneously hypertensive rats was found to be at the level of the control rats. Sen *et al* (1972) reported that in the spontaneously hypertensive rats the plasma renin activity is age-dependent. In young spontaneously hypertensive rats the plasma renin activity is increased (DeJong *et al* 1972 Sen *et al* 1972) but returns later to normal levels in rats with a body weight of over 200 g (Sen *et al* 1972) which corresponds to the weight of the rats of the present study.

The increased plasma renin activity does not seem to be essential for the hypertension in the pinealectomized rats. The beta adrenoceptor blocking drugs propranolol (Karppanen 1974) and tolamolol (Karppanen *et al* 1973 b) decreased the plasma renin activity of the pinealectomized rats. However only propranolol exerted an antihypertensive effect despite the fact that the plasma renin activity was depressed more by tolamolol. The synthesis of aldosterone in response to ACTH or DBA was diminished in the adrenal glands of the spontaneously hypertensive and pinealectomized rats as compared to the adrenal glands of the rats with renal hypertension. This may suggest a relative unimportance of the renin aldosterone system at least in the established spontaneous and pinealectomy induced hypertension as compared to the renal hypertension.

The sympathetic renal nerves are involved in the control of the release of renin from the juxtaglomerular cells (Birbari 1971). Both in the spontaneously hypertensive (Haessler *et al* 1972 van Zwieten 1973) and pinealectomized rats (Karppanen *et al* 1973 a) an increased sympathetic tone of central origin has been implicated in the pathogenesis of the hypertension. It has been suggested that the increased plasma renin activity of the pinealectomized rats may be due to the increased activity of the sympathetic renal nerves (Karppanen *et al* 1973 a). This might also be the case in the spontaneously hypertensive rats.

We suggest that the renin aldosterone system may be of crucial importance in the renal

TABLE I Effect of ACTH, DBA and 5HT on the release of aldosterone from rat adrenal quarters *in vitro*

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The values indicate the release of aldosterone in µg per mg N during the 7 h incubation. Means ± S.E. of 4–5 incubations are given.

PE = pinealectomized, RH = renal hypertensive, SH = spontaneously hypertensive rats.

<sup>a</sup>  $p < 0.05$  vs renal hypertensive rats.

<sup>b</sup>  $p < 0.01$  vs renal hypertensive rats.

<sup>c</sup>  $p < 0.001$  vs renal hypertensive rats.

### Plasma renin activity (Fig. 1)

5 weeks after the operation the plasma renin activity of the pinealectomized rats was increased by 35% ( $p < 0.01$ ). In the rats with renal or spontaneous hypertension the plasma renin activity did not differ from control values.

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### Plasma electrolytes (Table II)

The plasma sodium concentration was similar in all groups. In the rats with renal hypertension the plasma potassium was decreased ( $p < 0.001$ ) as compared to the unoperated control rats. In the pinealectomized and spontaneously hypertensive rats the plasma potassium concentration was unchanged.

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The electrolytes were determined 5 weeks after pinealectomy or the removal of the right kidney and weighing a figure of eight ligature around the left kidney.

$p < 0.001$  vs unoperated control rats.

## Optimal Parameters for Eliciting Cardio-Acceleration by Electrical Stimulation of the Ventromedial Hypothalamus

By

G JOHANSSON R KALIMO T PAAKKÖNEN and S RUUSUNEN

Received 19 December 1974

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### Abstract

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JOHANSSON G R KALIMO T PAAKKÖNEN and S RUUSUNEN *Optimal parameters for eliciting cardio-acceleration by electrical stimulation of the ventromedial hypothalamus* Acta physiol scand 1975 94 189-197

The ventromedial hypothalamus of the unrestrained cat was stimulated electrically through permanent electrodes. The stimulation parameters amplitude duration and frequency of the square wave pulses were combined to produce a  $\Delta 0^\circ$  increase of the resting heart rate value. The total electric charge for each parametric combination was calculated. The charge per stimulation (10 s) varied from about 1 to 100 micro-coulombs depending on the parametric combination used. The response could therefore not be expressed as a function of the charge but depended on the individual parametric combinations. The relation among the stimulation parameters and the total amount of electric charge of the pulses was estimated by the correlation technique. The results suggested that about 60% of the variation in the electric charge resulting in the constant response was due to the variation of the pulse repetition frequency. The optimal parameters for eliciting cardio acceleration in the cat by stimulation of the ventromedial hypothalamus seem to be pulse durations between 1 and 0.5 ms and pulse repetition frequencies below 100 P/s with pulse amplitude as the dependent variable.

**Key words:** Electrical stimulation parameters ventromedial hypothalamus cardio acceleration correlation analysis

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The parameters of electrical impulses applied to the hypothalamus have frequently been shown to influence the character and strength of the behavioral and physiological responses elicited (Keesey 1964 Cox and Valenstein 1969 Mogenson Gentil and Stevenson 1971 Wauquier Niemegeers and Gevers 1972 Johansson *et al* 1974). Some authors have suggested that parameters such as pulse repetition frequency (PRF) pulse duration (PD) and pulse amplitude (PA) can be subsumed under a common factor charge (Q) (Ward 1959 Keesey 1962 Huston Mills and Huston 1972). Keesey (1962) for example reported that the rate of hypothalamic self stimulation remained constant in spite of changes in the PRF and PA provided that the charge was constant. In contrast the longer the PD the more charge was required to maintain a certain rate of response.

Studies on parameters of hypothalamic stimulation have usually been carried out with



**hypertension** In the spontaneously hypertensive and pinealectomized rats the increased plasma renin activity may merely reflect an increased activity of the sympathetic nervous system which is suggested to be the main cause of hypertension in these rats

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**Histology** At the conclusion of the experiments the animals were killed their brains fixed in 10 neutral formaldehyde and the nuclei stained with Nissl stain (Romanis 1948). The positions of the electrode tips were then localized under a dissecting microscope. In 10 of the cats the electrodes had reached the ventromedial nucleus of the hypothalamus. In one the electrode had been situated in the dorsal nucleus of the hypothalamus and in one in the periventricular arcuate nucleus. Further, in one animal the site of the electrode tips could not be traced with certainty and in two no histological examination was made.

#### *ECG recording and basic heart rate (BHR)*

The electrodes (EEG electrodes of cup type) for the ECG recording were fixed in the chest of the cat with a rubber griddle. Electrode paste was used to increase the contact with the skin. The apparatus for the recordings was a Mingograf 4 II (Elema Schonander, Sweden).

The cat was put into the observation box for 15 min. after which 10-s ECG samples were recorded at one min intervals. The mean of 10 successive recordings in the reclining but alert animal with a variation of  $\pm 10\%$  at most was called the BHR.

#### *Control heart rate response (CR)*

After the BHR had been determined stimulations were applied at intervals of at least 5 min. It was checked that the ECG was always within the limits of the BHR before another stimulation was applied. Stimulation was only given when the animal was reclining but not asleep and never if it was moving. The train duration of the stimulation was 10 s. The heart rate response elicited was calculated 5 s after the beginning of stimulation. A 10% increase in the BHR was called the CR.

#### *Experimental situation 1*

In these experiments on 14 animals the CR was elicited by keeping the PD constant at 1 ms. PRFs of 10, 50, 100 and 500 Hz were combined with different PAs until the CR was obtained at each frequency. The PA was changed in steps at 5 min intervals as described above.

#### *Experimental situation 2*

PRFs of 10, 50, 100 and 500 Hz were combined stepwise with pulses of different durations. The PA was kept constant at a value which had been determined for each cat on the basis of pilot tests. It was the lowest PA which resulted in agonistic behavior (cf Nakao 1958 and Romanuk 1965) when the PRF was kept at 50 Hz and the PD at 1 ms. This value varied in the 6 animals used in these experiments from 0.1 to 0.5 mA (mean  $0.28 \pm 0.14$  mA).

#### *Experimental situation 3*

The PRF in these experiments was kept constant at 50 Hz and pulses of 3 different durations: 0.0, 0.2 and 2.0 ms were combined with varying PAs to evoke the CR. Eleven cats were stimulated in this experimental situation.

## Results

The electrical stimulation eliciting the CR was too weak to induce any visible vegetative or somato-motor responses in the cat. Stimulation intensities higher than those eliciting CR however resulted in agonistic behavior previously described by e.g. Nakao 1958, Romanuk 1965 and Johansson *et al.* (1974).

#### *Experimental situation 1*

The PA fell in a steep slope from PRF 10 through 50 to 100 Hz. When the PRF was 500 Hz, higher PAs were again needed to obtain the CR in 5 of the 14 cats. When analyzed by two-way analysis of variance (McNemar 1962, pp. 284-296) the differences between the means of the PA in the categories of PRF used were statistically significant ( $p < 0.001$ , Table 1 and Fig. 1). The monotonically decreasing trend of the PA-PRF curve appeared

TABLE I Means and standard deviations of pulse amplitude and total electric charge by pulse frequency  
PD = 10 ms N = 14

Pulse repetition frequency (Hz)	10		50		100		500	
	M	S D	M	S D	M	S D	M	S D
Pulse amplitude (mA)	0.23	0.11	0.13	0.05	0.09	0.06	0.17	0.10
Electric charge ( $\mu$ C)	2.70	1.09	8.33	4.79	12.88	6.43	104.34	52.3

Analysis of variance (a) Amplitude  $F=10.47$   $df=3, 39$   $p<0.01$  (b) Charge  $F=50.77$   $df=3, 39$   $p<0.01$

to be statistically significant ( $p<0.05$ ) in a directional nonparametric trend analysis (Ferguson 1965) with a monotonic relationship as a hypothesis.

Q corresponding to the parametric combinations used showed a tendency to increase with PRF ( $p<0.001$ , Fig. 1). The differences in the means of the Q (Table I) were also found to be statistically significant ( $p<0.001$ ).

#### Experimental situation 2 The PD-PRF curves

In spite of considerable deviation at a PRF of 10 Hz, the decreasing tendency of the PD was obvious up to PRF 50 (Fig. 2). In the PRF range 50–500 Hz differences in the PD were small. However, the differences among all the means were statistically significant ( $p<0.01$ ). The curve describing the interdependence of the PD and the PRF had a monotonically decreasing trend ( $p<0.025$ ). In the corresponding curve for Q the differences between the means were significant ( $p<0.01$ ) and so was the monotonically increasing trend ( $p<0.05$ ). The results of analysis of variance are shown in Table II.

#### Experimental situation 3 The PA-PD curve

The PA decreased from PD 0.02 through 0.2 to 2.0 ms, but the slope was steeper between PD 0.02 and 0.2 ms than from 0.2 ms on (Fig. 3). The longer the PD, the less the deviation of the PA. The differences in the means of the PA were statistically significant ( $p<0.001$ ) and the probability that the above described trend of these means was due to chance is almost zero ( $p=0.0005$ ). Concerning the Q-PD relationship the differences in the means of the Q were also significant ( $p<0.001$ , Table III) and so was the monotonically increasing trend of the Q with increasing PD ( $p<0.001$ , Fig. 3).

TABLE II Means and standard deviations of pulse duration and charge by pulse frequency  
PA = constant N = 6

Pulse repetition frequency (Hz)	10		50		100		500	
	M	S D	M	S D	M	S D	M	S D
Pulse duration (ms)	0.88	0.38	0.8	0.11	0.9	0.17	0.9	0.20
Electric charge ( $\mu$ C)	4.8	1.33	4.75	3.57	10.5	9.1	5.33	55.14

Analysis of variance (a) Duration  $F=9.19$   $df=3, 15$   $p=0.01$  (b) Charge  $F=4.88$   $df=3, 15$   $p=0.01$

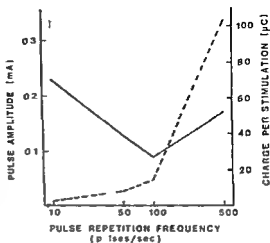


Fig. 1 Mean pulse amplitude (solid line surrounded by standard deviation) and electric charge per stimulation (dotted line) producing a 20% increase in the resting heart rate as functions of the pulse repetition frequency. Pulse duration = 1 ms.

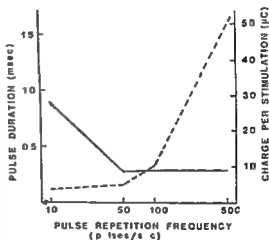


Fig. 2 Mean pulse duration (solid line surrounded by standard deviation) and electric charge per stimulation (dotted line) producing a 20% increase in the resting heart rate as functions of the pulse repetition frequency. Pulse amplitude = constant.

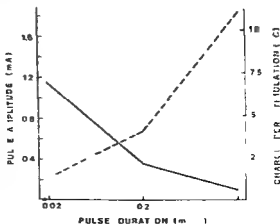


Fig. 3 Mean pulse amplitude (solid line surrounded by standard deviation) and electric charge per stimulation (dotted line) producing a 20% increase in the resting heart rate as functions of the pulse duration. Pulse repetition frequency = 100 Hz.

TABLE III Means and standard deviations of pulse amplitude and charge by pulse duration  
PRF = 50 Hz N = 11

Pulse duration (ms)	0.02		0.2		2.0	
	M	S.D.	M	S.D.	M	S.D.
Pulse amplitude (mA)	1.15	0.40	0.36	0.21	0.09	0.08
Electric charge ( $\mu$ C)	1.37	0.43	3.98	2.22	12.15	9.72

Analysis of variance (a) Amplitude  $F=83.46$   $df=2, 20$   $p<0.01$  (b) Charge  $F=13.47$   $df=2, 20$   $p<0.01$

### Correlation analysis of the data

The relation between the Q necessary to elicit the CR and the PA, PD and PRF as well as the interrelations of these three parameters were analyzed by the correlation technique. The product moment correlations were calculated across the 3 experimental situations in which the CR was produced. Each stimulation resulting in the CR was considered as an observation unit. The number of units was 113. The dependence of Q on each separate parameter partialing out the effect of the other two parameters was analyzed with partial and partial correlation techniques. Since the two techniques gave almost identical results the following considerations are based solely on the partial correlations.

Table IV lists the intercorrelations of PD, PRF, PA and Q. The correlations among the 3 first mentioned parameters were negative. 2 of them were low, but the correlation between PA and PD was quite high.

The correlation between Q and PRF was much stronger than the correlations of Q with the other parameters. Moreover, this was the only correlation that differed with statistical significance from zero ( $p=0.001$ , two-directional test). Although not differing significantly from zero, the negative correlation between PA and Q is notable.

After adjustment for PRF, the product moment correlation 0.13 between PD and Q was 0.23. This value differs with statistical significance from zero ( $p=0.05$ ) on the basis of applications of the  $t$  test, two-directionally, to the corresponding  $Z$  value of the partial correlation (Hays 1961, pp. 575-576). After the effect of PA was partialled out, the partial correlation was only 0.10. On the other hand, the corresponding partial correlation of the second order reached the value 0.31, which differs significantly from zero ( $p=0.01$ , two-directional test). The correlations of PRF with PD and PA were quite low, for which reason the product moment correlation 0.76 between the PRF and Q changed little after

TABLE IV Intercorrelations of stimulation parameters N = 113

	PD	PRF	PA	Q
Pulse duration (PD)	1			
Pulse repetition frequency (PRF)	0.03	1		
Pulse amplitude (PA)	0.56	0.14	1	
Electric charge (Q)	0.13	0.76	0.08	1

adjustments have been made for the other parameters. It reached the value 0.78 in the partial correlation analysis of the second order.

The initially low negative correlation between PA and Q reached a low positive value 0.04 after adjustment for PRF. The change in the coefficient was smaller after adjustment for PD, the partial correlation being -0.01. Essentially different results were obtained when the effects of both PRF and PD were partialled out. The partial correlation between PA and Q then reached the value 0.21, which differs statistically significantly from zero ( $p < 0.05$ , two directional test). However, the coefficient remained lower than the corresponding correlations between other parameters and Q.

### Discussion

The sensitivity of the heart rate to many overt stimuli as well as to movements of the body made strict control of the experimental conditions necessary. It was observed in our experiments that, for example, a rise from the lying position was enough to cause an increase in the heart rate of at least 20%. The effect of mere changes in skeletal muscle tone associated with hypothalamically induced behavior (see e.g. Hunsperger 1969) was therefore minimized by making recordings only when the animal was reclining, the heart rate then being in the range of the resting state. On the other hand, falling asleep caused a considerable decrement in the heart rate. Therefore, experiments were not made if the animal fell asleep. It has been shown that heart rate alterations due to stimulation are different for gradually rising than for steeply rising bursts of pulses (Hyde 1966). This source of error was excluded by always applying the stimuli separately with stepwise changes.

The considerable variation of the charge in the different parametric combinations capable of eliciting a constant heart rate reaction means that this stimulation response cannot be expressed as a function of the number of microcoulombs alone. This is not in keeping with the findings of Keesey (1962) concerning self-stimulated behavior. Keesey concluded that this behavior could be expressed as a function of the charge. It is notable that the charge in experimental situation 2, with a PRF of 500 Hz in combination with a constant PD of 1 ms, required about twice as much charge as was needed in situation 1, with the same PRF, in which PA was constant and PD the dependent variable. In the latter situation, the mean value of PD was 0.3 ms and the stimulation-off period was consequently longer. Thus, most of the difference in microcoulombs required in these special cases probably can be explained by differences in stimulation thresholds due to the refractory periods of the neuronal elements (cf. Abeles 1967). With PRFs of 100 Hz or less, the lower efficacy of an increase of the PD than of PA probably is due chiefly to other factors, such as differences in the size of the neuronal pool involved in eliciting the response (see e.g. Lullies 1961, Abeles 1967).

The correlation analysis showed that Q was essentially more sensitive to variation in PRF than in the other parameters. Actually, the results of the analysis (Table IV) suggest that about 60% of the variation in Q resulting in CR was due to variation in PRF. An increase in PD or PA in the ranges used was much less effective in eliciting the CR. When the results of the correlation analysis are interpreted, however, the way in which the

observation units were constructed in the present study must be taken into consideration. If other values had been used for the parameters that were kept constant or at fixed values in each experimental situation the correlations probably would have been different.

The charge required to evoke the constant heart rate response varied from 1.1  $\mu\text{C}$  to 104.3  $\mu\text{C}$  per stimulation depending on the parametric combination used (Table I II and III). The optimal combination when the aim was to obtain the lowest possible charge was  $\text{PD} \sim 0.02$  ms  $\text{PRF} \sim 50$  Hz and the mean of the dependent variable  $\text{PA} \sim 1.15$  mA (Table III). The least effective combination was  $\text{PD} \sim 1.0$  ms  $\text{PRF} \sim 500$  Hz, and the mean of the dependent  $\text{PA} \sim 0.17$  mA (Table I).

We suggest that the most physiological or optimal parameters for eliciting cardio-acceleration in the cat by stimulation of the ventromedial hypothalamus are PDs between 0.1 and 0.5 ms and PRFs below 100 Hz with PA as the dependent variable. Shorter PDs may require values of PA so great as to cause injury (Mickle 1961). The results do not permit generalizations about the parameters suitable for eliciting the numerous other autonomic and behavioral responses that can be elicited from the same brain area but may help in the choice of them.

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## Facilitation from Contralateral Primary Afferents of Inter-neuronal Transmission in the Ia Inhibitory Pathway to Motoneurones

By

L. FEDINA<sup>1</sup> H. HULTBORN and M. ILLERT<sup>2</sup>

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### Abstract

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The action of volleys in contralateral primary afferents on transmission in the Ia inhibitory pathways to motoneurones was investigated with intracellular recording from motoneurons. Ia IPSPs in flexor as well as most extensor motoneurons were regularly facilitated by volleys in contralateral high threshold muscle cutaneous and joint afferents in spinal cats under chloralose anaesthesia. In decerebrate cats with a low portion of transmission in Ia inhibitory pathways was not facilitated but rather depressed by volleys in the afferents. The recurrent effects from motor axon collaterals were investigated in inhibitory transmission from different contralateral afferents to motoneurons. Previous investigations have shown that the motoneurons mediating the reciprocal Ia inhibition receive recurrent inhibition. A motor axon collateral and Renshaw cells. Now a strong positive correlation was revealed between recurrent depression of IPSPs evoked from different contralateral afferents and facilitation of Ia IPSPs by the same afferent. These results suggest that the recurrent depression of IPSPs from different contralateral primary afferents is due to excitatory convergence onto the Ia inhibitory interneurons which then produce the IPSP evoked in the motoneuron from these afferents.

In a preceding paper Fedina and Hultborn (1972) analysed the effects on the interneuronal transmission in Ia inhibitory pathways to motoneurons by volleys in ipsilateral segmental afferents. It was concluded that motoneurons in the Ia inhibitory pathways to flexor as well as extensor motoneurons receive excitatory convergence from ipsilateral flexor reflex afferents (FRA) in spinal chloralose anaesthetized cats. In decerebrate cats with a low portion of transmission (Holmqvist and Lundberg 1961) the transmission in that excitatory FRA pathway was blocked but an excitatory convergence from an apparently separate pathway from low threshold cutaneous afferents was still present.

Antidromic impulses in motoneurons were earlier shown (Hultborn, Jankowska and Lundström 1971a, b) to accept transmission in the Ia inhibitory pathway to motoneurons by

<sup>1</sup> Present address: Institute for Experimental Medicine, 113 Budapest VIII Hunary

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a postsynaptic inhibition of the Ia inhibitory interneurons evoked via  $\alpha$  motor axon collaterals and Renshaw cells Fedina and Hultborn (1972 cf also Hultborn *et al* 1971 a) investigated also the recurrent effects from motor axon collaterals on inhibitory transmission to motoneurons from other ipsilateral afferents. A strong positive correlation was revealed between the recurrent depression of IPSPs evoked from different ipsilateral afferents and the ability of the same afferent volleys to facilitate Ia IPSPs. This was taken to indicate that recurrent depression of IPSPs from different ipsilateral primary afferents reflect their excitatory convergence on the Ia inhibitory interneurons.

The present investigation analyses in a similar way the facilitation of transmission in Ia inhibitory pathways to motoneurons by volleys in contralateral segmental afferents in different types of preparation. The ability to evoke such a facilitation was then compared with the susceptibility to recurrent depression of the IPSPs evoked in motoneurons by the same contralateral volleys. The systematic comparison has revealed a strong positive correlation between these events. Our results thus support the suggestion by Hultborn *et al* (1971 a) that the recurrent inhibition of interneurons in afferent pathways to motoneurons is restricted to those mediating the reciprocal Ia inhibition to motoneurons.

### Methods

Most of the present material (derived from 6 cats totally) was collected from the same experiments presented in a preceding paper (Fedina and Hultborn 1972) dealing with the effect on transmission in the Ia inhibitory pathways by volleys in ipsilateral afferents. The maintenance of the preparation and the experimental procedures followed that described in previous papers (Fedina and Hultborn 1972; Hultborn and Udo 1977). Ipsilateral and contralateral muscle and cutaneous nerves and a nerve branch from the knee joint were dissected and mounted on electrodes for stimulation. These nerves are listed under Abbreviations.

Four different preparations were used (see Fedina and Hultborn 1972): I) unanaesthetized decerebrate decerebellate cats with a low pontine lesion—henceforth called unanaesthetized low pontine preparation (see Holmgvist and Lundberg 1961); II) same as in I) but with chloralose anesthesia (60 mg/kg); III) unanaesthetized low spinal animals which were either decerebrated or anaemically decorticized; IV) spinal animals with chloralose anaesthesia.

The technique of stimulation of peripheral nerves and of intracellular recording from motoneurons has been described (Hultborn and Udo 1977).

**Recording system.** In addition to the conventional recording of intracellular events the recorded potentials were fed to an averaging computer (CAT 1000). In order to minimize errors due to slow random variations in the potentials the unconditioned and conditioned responses were alternated and fed to different parts of the computer memory. The averaged responses were displayed on an oscilloscope for photographing. For a quantitative evaluation of the action of contralateral volleys on ipsilateral Ia IPSPs the use of a digitizing system (HP 9330) proved very helpful (cf Fig 5 and 6). Encoded records of the intracellular potentials were traced with an analogic digital converter. The digital values which were stored in a computer memory were used for various calculations. This it was possible to subtract the different responses from one another and to take the integral of arbitrary parts of the recorded potential. These could be compared according to the conditioning test system.

**Abbreviations.** The following abbreviations are used: ant. ro. biceps and semimembranosus, ABSm; deep peroneus (without cutaneous and extensor digitorum brevis branches), DPgt; m. s. d. soleus, GS; posterior biceps and semitendinosus, PBSt; extensor digitorum longus, EDL; flexor digitorum and hallucis longus, FDL; hamstring nerve (consisting of PBSt and ABSm), Hpn; peroneal nerve, plantaris, PI; quadriceps, Qsart; sartorius, Sst; sur. tibialis (flexing biceps to popliteus), tib. flex. post. or. and flexor digitorum and hallucis longus muscles, Tbflex; afferents, FR; afferent, FRa; afferent, FRa; postsynaptic potential, PSP; excitatory postsynaptic potential, EPSP; inhibitory postsynaptic potential, IPSP; recurrent inhibitory postsynaptic potential, RIPSP; dorsal root potential, DRP; primary afferent depolarization, PAD; ipsilateral and contralateral, i.a. and c.a.

**TABLE 1** Facilitation of Ia IPSPs by volleys in contralateral high threshold afferents. The species of the motoneurons investigated are indicated to the left and the figures in parentheses indicate the number of cells tested. The different preparations are given in the upper horizontal row, column 1-4. Note that the preparations labelled 'decerebrate' refer to decerebrate decerebellate cats with a low pontine lesion as described in Methods. In each preparation the effect of stimulation of contralateral high threshold muscle afferents (coH), contralateral high threshold cutaneous afferents (coSur) and contralateral high threshold joint afferents (coJ) are listed separately. The figures presented for each nerve show the number of motoneurons in which a spatial facilitation was established of the total number of motoneurons tested in that combination (convergence/total). The sum of motoneurons recorded in the different preparations is greater than the number given in the parentheses on the left side since often different nerves were tested in one motoneuron. In addition, sometimes one motoneuron was tested in different preparations (cf. Fig. 6 and 8).

		1 Decerebrate unanesthetized			2 Decerebrate chloralose			3 Spinal unanesthetized			4 Spinal chloralose		
		coH	coSur	coJ	coH	coSur	coJ	coH	coSur	coJ	coH	coSur	coJ
Sart (7)	Convergence/total	—	—	—	—	—	—	—	—	—	6/6	4/4	—
PBSi (67)	Convergence total	0/10	1/10	—	0/9	0/3	—	2/9	1/7	—	77/30	47/3	3/3
DP (11)	Convergence total	—	—	—	1/1	1/1	—	0/1	—	—	3/5	6/6	3/3
ABSm (19)	Convergence total	—	—	—	—	—	—	0/4	—	—	13/13	10/11	1/1
Q (38)	Convergence/total	0/8	0/1	—	0/2	0/1	—	0/5	1/3	—	10/15	9/9	—
G S (16)	Convergence/total	—	—	—	0/1	0/1	—	0/4	0/1	0/1	0/1*	1/9	0/4

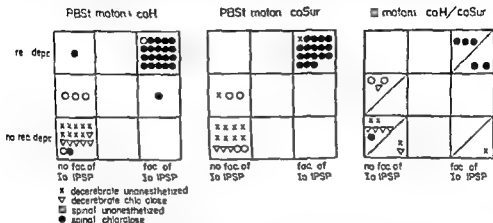
## Results

The action of volleys in contralateral high threshold muscle afferents, high threshold joint afferents and cutaneous afferents upon transmission in the ipsilateral Ia inhibitory pathways to different species of lumbar motoneurons are summarized in Table 1. In this table only the effects of volleys in coH, coSur and coJ are presented. Other contralateral nerves (peroneal, tibial and G S nerves) were also occasionally tested but no qualitative differences were found with the results in Table 1. The recurrent control by volleys in ipsilateral motor axon collaterals on transmission in the inhibitory pathways from the contralateral afferents to the motoneurons was also tested. All ventral roots (L5-S1) besides those evoking a recurrent IPSP in the recorded motoneuron were then used for conditioning stimulation. It was always checked that the same conditioning volley depressed Ia IPSPs in the same cell. This recurrent control was then correlated in individual motoneurons with the facilitation of the ipsilateral Ia inhibition evoked by the same contralateral afferents. Table 2 summarizes this correlation for the PBSi and Q motoneurons tested.

### 1. Spinal chloralose anesthetized preparation

**Flexors.** Fig. 1 illustrates two knee flexor PBSi motoneurons (A-F, □, I). The test Ia IPSPs from Q (second trace in A, D, G) were conditioned by stimulation of nerves from the contralateral side. When the conditioning contralateral volley itself evoked an IPSP (which was the usual case) the test Ia IPSP was ordinarily placed on its decay phase (as in Fig. 1 A, D and G) to avoid the problems of a nonlinear summation of IPSPs and a conductance increase caused by the conditioning volley. High threshold cutaneous afferents (coSur, 0-11).

**TABLE II** Comparison between the susceptibility to recurrent depression of IPSPs from contralateral high threshold muscle afferents (coH) and contralateral high threshold cutaneous afferents (coSur) and the ability by volleys in the same afferents to facilitate transmission in the 1a inhibitory pathway to PBSt and Q motor nuclei. For each individual motoneurone the susceptibility to recurrent depression of IPSPs from the contralateral high threshold afferents has been plotted against the ability by volleys in the same afferents to facilitate a test 1a IPSP. Different symbols indicate the type of preparation. Note that the preparations labelled decerebrate refer to decerebrate decerebrate cats with a low pontine lesion. Notice that the "middle" groups (both at the abscissa and ordinate) means that it was not possible to decide if the effect was present or absent — no decision



high threshold muscle afferents (coH 25 T) and high threshold joint afferents (coj 50 T) effectively enhanced the test 1a IPSPs (third trace in the respective sets). This facilitation proves an excitatory action from these contralateral afferents on ipsilateral 1a inhibitory interneurons which project to PBSt motoneurons. The facilitatory action of volleys in contralateral muscle and cutaneous afferents on transmission in the 1a inhibitory pathways sometimes became evident with stimulation strengths as low as 2.5–6 T (i.e. gr 1 muscle afferents never contributed). However a strength of more than 10 T was often required and was always necessary with contralateral joint afferents. In addition it proved sometimes useful to apply a short train (2–3 shocks) to demonstrate the facilitation. Usually the facilitation of 1a IPSPs increased with increasing stimulation strength up to about 50 T. The contralateral muscle afferents (irrespective of muscle function) cutaneous and joint afferents facilitated the ipsilateral 1a inhibitory transmission without qualitative differences. It is therefore reasonable to assume that the observed facilitation of the 1a IPSPs can be ascribed to an activation of a contralateral FRA pathway (R. M. Eccles and Lundberg 1959 and Holmqvist 1961).

A conditioning stimulation of L5 and L6 ventral roots depressed the facilitated 1a IPSP (Fig. 1 lowermost traces in A–G). In some cases it was nearly abolished by the ventral root stimulation (for example Fig. 1 D). This demonstrates the convergence of excitation from contralateral FRA and inhibition from motor axon collaterals on the same 1a inhibitory interneurons (see Hultborn and Udo 1972, pp. 97–98 for a detailed description of this technique). It should be noticed that the FRA actions described here must be mediated by segmental pathways since the spinal cord was transected at low thoracic level.

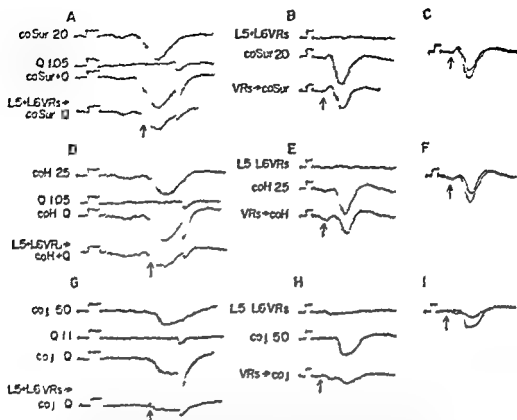


Fig. 1. Facilitation of Ia IPSPs in knee flexor motoneurons (PBSt) by volleys in contralateral high threshold afferents and concomitant recurrent depression of the IPSPs evoked from the same contralateral high threshold afferents. (Chloralose anesthetized spinal cat. Two motoneurons (A, D, G). All traces are averaged intracellular responses. In this and following pictures the positivity is upwards for the intracellular cord. Calibration pulses: 1 mV, 4 ms. The motoneuron of A, D, G was depolarized by a current of 70 nA. The left column (A, D, G) shows the conditioning effect by volleys in coSur (A), coH (D) and coJ (G) on the Ia IPSPs. The stimulation strength is indicated in multiples of threshold for the fastest afferent fibres. The recurrent effect of VR stimulation (indicated by a row) on the conditioned Ia IPSPs is tested in the middle column (B, E, H). The effect of VR stimulation on the IPSPs evoked from the respective contralateral nerves is illustrated. The conditioned and unconditioned contralateral IPSPs are superimposed in the right column (C, F, I).

The records B, E and H (Fig. 1) illustrate the action of a ventral root stimulation on the IPSPs which were evoked from the coJ RA. The superposition of the conditioned and unconditioned traces in C, F and I shows an effective depression of these IPSPs by a preceding ventral root stimulation. It is noted that the recurrently depressed parts of IPSPs in fact are mediated by Ia inhibitory interneurons; this finding would indicate that at least to some extent the Ia inhibitory interneurons relay the IPSPs evoked in PBSt motoneurons by contralateral FRA (see Discussion).

The facilitation of Ia IPSP by coJ RA has been tested in different species of flexor motoneurons. It can be seen from Table 1 that in the spinal preparation anesthetized with chloralose the facilitation was present in hip, knee and ankle flexor motoneurons (Fig. 2).

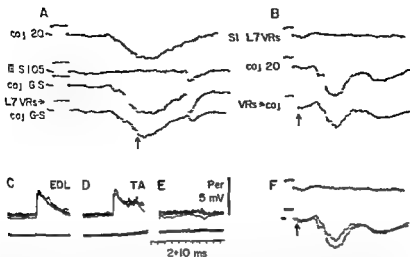


Fig 2 Facilitation of the Ia IPSP in a pretibial flexor motoneurone (DP) by volleys in the contralateral joint afferents and concomitant recurrent depression of the IPSP evoked from the same contralateral joint afferents. Chloralose anaesthetized spinal cat. The motoneurone was depolarized by a current of 70 nA. In C-E showing the identification of the neurone the upper traces are intracellular potentials and the lower traces are recorded from the L7 S1 dorsal root entry zone. In the surface lead in this and the following figures the negativity is upwards. Time and voltage calibration for C-E are indicated in E. The traces in A, B and F are averaged intracellular responses. Calibration pulses: 1 mV, 4 ms. The time of stimulation of VRs is marked by arrows. A shows the conditioning effect of coj stimulation on the Ia IPSP. In the lowermost trace L7 S1 VRs are stimulated additionally. In B the recurrent effect of VR stimulation on the coj IPSP is shown. The conditioned and unconditioned IPSPs are superimposed in F.

exemplifies the effects found in pretibial flexor motoneurons. In a DP motoneurone (identification in C-E) the Ia IPSP from G-S (1.05 T) was enhanced by conditioning stimulation of coj (20 T, A) and the IPSP by the coj volley was depressed by a conditioning ventral root volley (B, F) just as illustrated for PBSt motoneurons in Fig 1. Facilitation of Ia IPSPs was observed in 10 out of 11 investigated DP motoneurons and was also noticed in one Per motoneurone (not included in Table 1). Two of the DP motoneurons were identified antidromically from the TA nerve (in an experiment with an intact L6 ventral root). However, although a facilitation of the Ia IPSPs in DP motoneurons was found regularly in our limited material (Table 1) it was obvious that this facilitation was not as pronounced as that found in knee flexor motoneurons.

The records of Fig 3 illustrate the time course of the facilitation of a Ia IPSP evoked by a conditioning stimulation of the coSur nerve. In a hip flexor Sart motoneurone stimulation of the nerve from the ABSm muscle evoked a minute test IPSP (A, faster sweep speed in the lower two traces). A conditioning volley in the coSur preceding the test stimuli with different time intervals facilitated the test IPSP (A-D). A conditioning ventral root stimulation decreased the facilitated IPSP (E). As can be seen from the specimen records (B-D) the degree of facilitation was dependent on the interval between conditioning and test stimulation. The full time course of the facilitation in this cell is shown in F. The latency and time course of the facilitation of Ia IPSPs from contralateral  $h_{th}$  threshold muscle

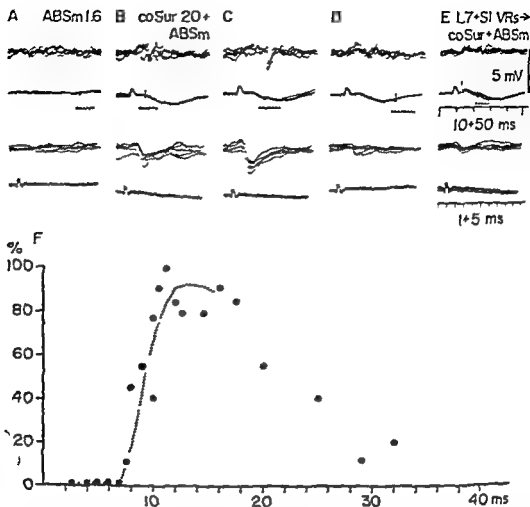


FIG. 3. Time course of the facilitatory action by contralateral high threshold cutaneous afferents on a Ia IPSP in a Sarii motoneurone. Chloralose anaesthetized spinal cat. The motoneurone was depolarized by a current of 100 nA. In A E the conditioned and unconditioned Ia IPSPs were recorded simultaneously at a slow and a fast sweep speed. The dashed lines below the upper slow records show the parts which are expanded in the lower records. In the respective pairs the upper traces are intracellular responses and the lower traces are recorded from the L6 dorsal root entry zone. Time calibration as indicated in E. The voltage calibration refers to the intracellular potentials. A-D give specimen records of the facilitatory time course. In E the facilitated IPSP was abolished by lowering a conditioning volley in S1 L7 VRs. In F the time course of the facilitatory effect is plotted. The abscissa gives the interval between the arrival of the fastest conducting fibres (measured by the initial points in the surface leads of the conditioning and test volley). Since the test stimulation elicited no Ia IPSP (A) the ordinate gives the amplitude of the facilitated Ia IPSPs as percent of the maximal Ia IPSP (11 msec conditioning test interval) which was set to 100.

afferents is approximately the same as illustrated in Fig. 3 for cutaneous afferents. However the threshold for the "direct" postsynaptic potential by coFRA stimulation was often lower than the threshold to evoke a facilitation of Ia IPSPs. This made it difficult and often impossible to investigate the time course. In a forthcoming paper using direct recording of the

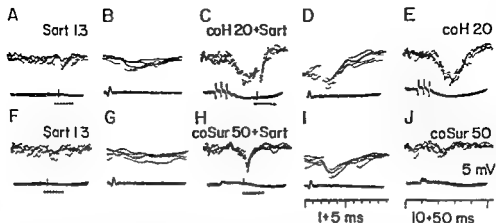


Fig 4 Facilitation of an Ia IPSP in a hip extensor motoneurone (ABSm) by volleys in contralateral high threshold muscle and cutaneous afferents. Chloralose anaesthetized spinal cat. The motoneurone was depolarized by a current of 70 nA. The upper traces are intracellular responses. The lower traces are recorded from L7 dorsal root entry zone. The dashed lines below the slow traces show the parts which were expanded in the neighbouring records. Time calibration for the slow records in J for the fast records in I. The voltage calibration refers to the intracellular potentials.

interneurones mediating reciprocal Ia inhibition (Hultborn, Illert and Santini, to be published) the latency and time course of the coFRA actions will be further illustrated.

**Extensors.** In hip, knee and ankle extensor motoneurones excitation of contralateral high threshold afferents elicited EPSPs predominantly. However, current passage through the microelectrode revealed that volleys in the coFRA usually evoked an IPSP in addition to the dominating EPSP. Following depolarization of the motoneuronal membrane it was therefore often possible to investigate in extensor motoneurones the action of coFRA upon the Ia inhibitory transmission as well as the recurrent control of the IPSPs evoked from the same contralateral afferents.

Fig 4 illustrates an hip extensor ABsm motoneurone in which the membrane had been depolarized. Volleys in contralateral high threshold afferents of muscular (coH 20 T) and also to some extent of cutaneous (coSur 50 T) origin evoked IPSPs (E and J). When the Ia IPSP was preceded by the contralateral FRA volleys it was effectively facilitated (cf A-D, F-I). The increase of the test Ia IPSP appears particularly clear in the fast traces of D and I. They suggest a convergence of coFRA excitation on the Ia inhibitory interneurones projecting to hip extensor muscles.

Fig 5 illustrates a representative knee extensor Q motoneurone. In the depolarized cell stimulation of the coSur nerve elicited an IPSP (upper trace in A). The Ia IPSP evoked by PBSt (second trace) was conditioned by a volley in the coSur (third trace). The dashed line in A indicates the part of the traces which is reproduced in B using an expanded time scale. Superposition of the traces (see further in legend) shows that the volley in the coSur facilitated the Ia IPSP. The records in C and D show that the IPSP evoked by the volley which facilitated the test Ia IPSP was depressed by antidromic volleys in L7 and S1 ventral roots (cf the superimposed traces of the unconditioned and conditioned IPSP in D).



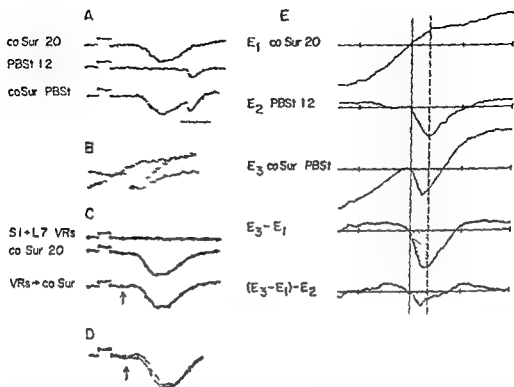


Fig. 5. Comparison of susceptibility to recurrent depression of contralateral high threshold cutaneous IPSPs and ability by volleys in the same contralateral afferents to facilitate Ia IPSPs in a Q motoneurone chloralose anaesthetized spinal cat. The motoneurone was depolarized by a current of 100 nA. The traces are directed by arrows. A shows the conditioning effect of a contralateral high threshold cutaneous volley (co Sur) on the Ia IPSP. The dashed line below the records indicates the part of the traces which is shown at an expanded time scale in B. The averaged intracellular responses after conditioning and conditioning test stimulation are arranged in such a manner that the contralateral high threshold IPSPs are superimposed. In addition to evaluate a possible facilitation of the Ia IPSP the unconditioned Ia IPSP is superimposed on the peak of the conditioned Ia IPSP. E shows the quantitative analysis of this facilitation. Further description in the text. In C the effect of VR stimulation on the IPSP evoked from the contralateral high threshold cutaneous afferents is illustrated. The conditioned and unconditioned IPSPs are superimposed in D.

Stimulation of coFRA facilitated the transmission in the Ia inhibitory pathway in most Q motoneurons tested. However, Fig. 5 illustrates one of the more evident examples of this excitatory convergence from coFRA on Ia inhibitory interneurons impinging on Q motoneurons—the action was usually weaker. As can be seen in Fig. 5 A, the estimation of the amount of facilitation was therefore rendered more difficult since the test Ia IPSP had to be placed on the decay phase of the IPSP elicited by the contralateral nerve. Therefore the potential evoked by the conditioning stimulation had to be subtracted from the response which was due to the combined conditioning test stimulation.

Fig. 5 E shows the subtraction procedure as it has been performed in the Q cell of A. The curves of A were traced using an analogue-digital converter (cf. Methods). E<sub>1</sub> and E<sub>2</sub> show the unconditioned and conditioned Ia IPSP. E<sub>3</sub> shows the corresponding period of the

IPSP evoked by the conditioning coSur stimulation. The reference point at the beginning of the unconditioned Ia IPSP is indicated in each trace by the crossing of the solid horizontal and vertical lines. The fourth trace gives the calculated potential after subtracting  $E_1$  from  $E_2$ , thus revealing the facilitated Ia IPSP in isolation. The component which is added to the test Ia IPSP ( $E_2$ ) by the conditioning stimulation is shown in the lowermost trace which is formed by a further subtraction of the unconditioned Ia IPSP ( $E_1$ ) from the isolated conditioned Ia IPSP ( $E_2 - E_1$ ). To estimate the percent change of the Ia IPSP effectuated by the conditioning stimulation the potentials were integrated over a limited period from the beginning of the unconditioned Ia IPSP to its peak (indicated by the broken line). In the fourth trace the hatched area gives the integral of the conditioned Ia IPSP after subtracting  $E_1$  from  $E_2$ . This area was compared with the integral of the unconditioned IPSP which was set to 100% (dotted area in  $E_2$ ). The hatched part in the lowermost trace marks the integral of the area which was added to the test Ia IPSP by the conditioning stimulation. In this cell the conditioned Ia IPSP ( $E_2 - E_1$ ) was facilitated to 148% of the unconditioned test IPSP.

All knee and ankle extensor motoneurons have been analysed according to this procedure. Table I reveals a difference between these two groups of motoneurons. Whereas in the spinal cat with chloralose anaesthesia the Ia IPSP to Q motoneurons was facilitated regularly by contralateral high threshold muscle and cutaneous afferents this facilitation was not found in the Ia inhibitory pathway to G-S motoneurons in the present conditions (cf. Discussion).

## 2 Spinal unanaesthetized preparation

In the spinal unanaesthetized preparation volleys in contralateral high threshold afferents only rarely facilitated the Ia IPSPs (Table I). This finding applies as well to flexor as to extensor motoneurons. Fig. 6 illustrates a flexor motoneuron (PBSt) in a spinal preparation. In the unanaesthetized state (A-B) the Ia IPSP from Q (11 T) was conditioned by stimulation of coH (20 T). The averaged records of A and the arithmetic procedure in B reveal that the conditioning stimulation had no effect on the test Ia IPSP. However, after intravenous injection of chloralose (60 mg/kg) a facilitation of the Ia IPSP became evident. Now volleys in the coH greatly enhanced the test Ia IPSP (C and D). This proves the convergence of contralateral high threshold muscle afferents on ipsilateral Ia interneurons. As an additional indication of this excitatory convergence the stimulation of the coH now decreased the time to peak of the test Ia IPSP (see  $D_1$  and  $D - D_1$ ).

## 3 Decerebrate cats with a low pontine lesion (low pontine preparation)

A suppression of coFRA actions is seen in the decerebrate state, but after a medial low pontine lesion there is a release of inhibitory paths from coFRA to both flexor and extensor motoneurons, while transmission in the excitatory paths is still suppressed (Holmqvist 1961; cf. Holmqvist and Lundberg 1961 for the ipsilateral FRA). Originally it was therefore thought that this latter preparation, henceforth referred to as the low pontine preparation, would offer excellent possibilities for analysis of the inhibitory FRA pathways to motoneurons and its presumed mediation via the Ia inhibitory interneurons. However,

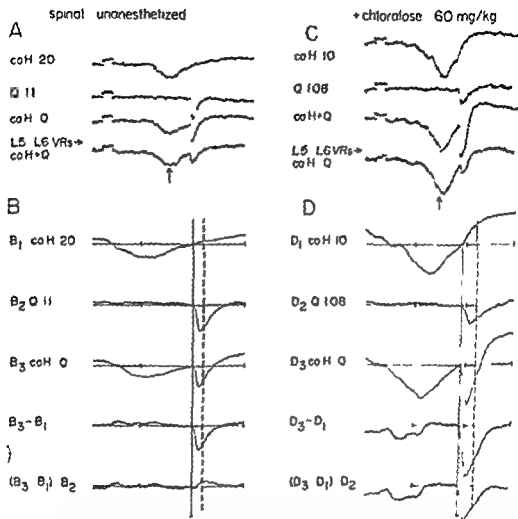


Fig. 1. Action of flexor digitorum profundus muscle afferents on Ia IPSPs in the spinotomedal preparation. (A) B1 and after administration of chloralose (C, D). The PBSt motoneurone was depolarized by 50 nA. A and C are averaged intracellular responses (calibration pulses 1 mV, 4 ms). Stimulus artefacts are marked by arrows. In A and C the conditioning effect by volleys in coH on the Ia IPSP is shown. The lowermost traces illustrate the effect of an additional VR stimulation on the conditioning effect. In B and D the conditioning effect of the conditioning stimulation on the Ia IPSP is shown. Further details are given in the text.

preceding investigation on ipsilateral FRA pathways (Fidina and Hultborn 1972) surprisingly revealed that although volleys in ipsilateral FRA evoke IPSPs in all motoneurons in such preparations there were never any sign of facilitation of Ia IPSPs.

Fig. 7A-C exemplifies the action of contralateral high threshold muscle afferents in an unanaesthetized low pontine preparation. In a PBSt motoneurone the Ia IPSP was elicited by stimulation of the Q nerve (Fig. 7A). Conditioning stimulation of coH (50 Hz) which evoked an IPSP in the motoneurone did not increase but rather decreased the amplitude

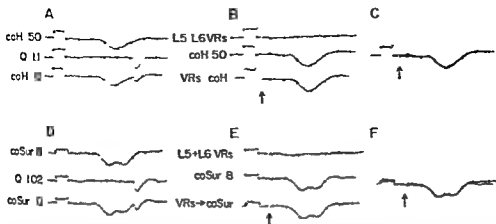


Fig. 7 Comparison of susceptibility to recurrent depression of contralateral high threshold muscle and cutaneous IPSPs and ability by volleys in the same contralateral afferents to facilitate Ia IPSPs in the decerebrate unanaesthetized state. Two PBSt motoneurons (A-C 60 nA depolarized; D-F 56 nA depolarized) were recorded in the same animal. All traces are averaged intracellular responses. Calibration pulses: 1 mV for A-C, 4 mV for D-F, 4 ms. The stimulation of VRs is indicated by arrows. A and D show the conditioning effect of high threshold volleys in coH (A) and coSur (D) on the Ia IPSPs. In B and E the effect of VR stimulation on the IPSPs evoked from the contralateral nerves is illustrated. The conditioned and unconditioned contralateral IPSPs are superimposed in C and F.

of the Ia IPSP. In B and C (Fig. 7) the recurrent control of the IPSP evoked by the contralateral afferents was investigated. Antidromic volleys in the L5-L6 ventral roots had no effect on this contralateral IPSP as can clearly be seen from the superimposed conditioned and unconditioned traces in C. Similarly as in flexor motoneurons it was found that volleys in high threshold muscle afferents did not facilitate the Ia IPSP in extensor motoneurons (Table I) and that IPSPs evoked from the same afferents were unaffected by antidromic volleys (Table II).

Fedina and Hultborn (1972) reported that in the low pontine preparation ipsilateral cutaneous afferents of low threshold did facilitate Ia IPSPs, thus in contrast with the high threshold muscle and joint afferents. However, as can be seen from Table I in the low pontine preparations the contralateral cutaneous afferents did not act independently from high threshold muscle afferents. This is illustrated for a PBSt motoneuron in Fig. 7. Records in E-F (see also Table II) show that the coSur IPSP itself was unaffected by conditioning antidromic volleys in L5 and L6 ventral roots which effectively depressed the Ia IPSP in the same cell (not shown). The lack of recurrent depression of inhibition from contralateral skin afferents to PBSt and Q motor nuclei was also confirmed by the use of monosynaptic test reflexes (6 cats). This was in sharp contrast to the effective recurrent depression of ipsilateral cutaneous inhibition of monosynaptic test reflexes in the same preparations (cf. Fig. 10 in Fedina and Hultborn 1972).

In the unanaesthetized spinal state the facilitation of Ia IPSPs by contralateral FRA was rather infrequent but was observed regularly after administration of chloralose (see above). Thus the question arose whether the failure to facilitate Ia IPSP in the unanaesthetized low pontine cat was due to the absence of the chloralose anaesthesia. However, a com-

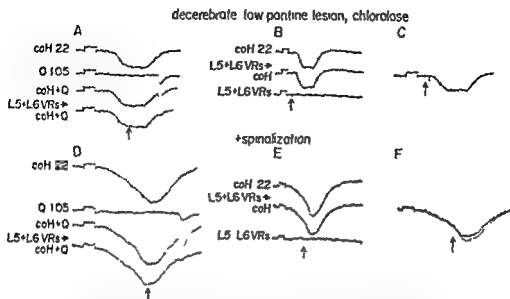


Fig. 8. Comparison of susceptibility to recurrent depression of contralateral high threshold muscle IPSPs and ability by volleys in the same contralateral afferents to facilitate Ia IPSPs in the decerebrate and the spinal state with chloralose anaesthesia. The PBSt motoneurone recorded in the decerebrate state with a low pontine lesion (A-C) and after subsequent spinalization (D-F) was depolarized by 70 mV. All traces are averaged intracellular responses. Calibration pulses: 0.5 mV, 4 ms. The stimulation of VRs is indicated by arrows. A and D show the conditioning effect of high threshold volleys in coH on the Ia IPSP. The recurrent effect of VR stimulation is shown in the lowermost traces. In B and E the effect of VR stimulation on the IPSPs evoked from the coH is illustrated. The conditioned and unconditioned contralateral IPSPs are superimposed in C and F.

Comparison between column 1 and column 2 in Table 1 reveals that in the low pontine preparation the additional presence of chloralose had no influence on the coFRA action in Ia IPSPs. Fig. 8 illustrates this point for a PBSt motoneurone which was first recorded in the chloralose anaesthetized low pontine state and then continuously during and after a complete spinalization. In the low pontine state (A-C) the Ia IPSP was not influenced by the conditioning stimulation of contralateral high threshold muscle afferents (A). B and C demonstrate that in addition the transmission in the inhibitory path from coH to the motoneurone was not controlled by antidromic volleys in motor axon collaterals. After spinalization (D-F) however, the Ia IPSP was effectively facilitated by the same conditioning volley in the coH (D). The additional stimulation of L5, L6 VRs nearly abolished the facilitated Ia IPSP, thus demonstrating the convergence of coFRA and recurrent inhibition on the same Ia inhibitory interneurons. Furthermore, now the transmission in the inhibitory pathway from coH to motoneurons was depressed by volleys in the recurrent motor axon collaterals (E-F). The superposition of the unconditioned and conditioned traces in F shows a distinct depression of the conditioned IPSP. These results clearly demonstrate that in the low pontine preparation (unanaesthetized or with chloralose anaesthesia) the transmission in the excitatory path from coH to Ia inhibitory interneurons is depressed.

In the decerebrate state with a low pontine lesion it was thus a consistent finding that volleys in the contralateral nerves did not facilitate the test Ia IPSP (Table 1). On the con-

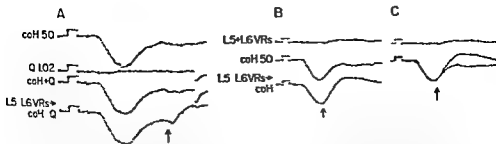


Fig 9 Late facilitation of a Ia IPSP by conditioning volleys in contralateral high threshold muscle afferents in the decerebrate unanaesthetized state and concomitant recurrent depression of the late parts of the IPSP evoked by the same contralateral muscle nerve. The PBSt motoneurone was depolarized by 17 nA. All traces are averaged intracellular responses. Calibration pulses 1 mV 4 ms. Stimulation of VRs marked by arrows. In A the conditioning effect of volleys in coH on the Ia IPSP is shown. The lowermost trace illustrates the effect of an additional stimulation of VRs. In B the effect of VR stimulation on the IPSP evoked from the contralateral high threshold muscle afferents is illustrated. The conditioned and unconditioned IPSPs are superimposed in C.

trary they often reduced the amplitude of the Ia IPSPs (Fig 7 A and D). By measuring the input resistance of the motoneurons (Eide 1968) it was often possible to exclude that a conductance change in the motoneuronal membrane was responsible for the decreased amplitude of the IPSP. Further it was always ascertained that the depression of the test Ia IPSP was not caused by recurrent inhibition secondary to a discharge of motoneurons following the coFRA volley (no discharge was elicited in the ventral roots). Thus the results indicate that in the low pontine preparation volleys in contralateral high threshold muscle and cutaneous afferents may depress the transmission in the Ia inhibitory pathways to motoneurons. The inhibition from coFRA of the interneurons mediating the reciprocal Ia inhibition will be dealt with in more detail in a forthcoming paper using direct recording from these interneurons (Hultborn, Illert and Santini, to be published).

In two exceptional unanaesthetized low pontine preparations stimulation of coFRA elicited two phases of inhibition. The early IPSP (with an approximate central latency—measured from the group I input volley—of about 5–6 msec) corresponds to the IPSPs illustrated in Fig 7 A–C, D–F and Fig 8 A. These “early” coFRA IPSPs were never depressed by conditioning volleys in the ventral roots and there was no facilitation of Ia transmission during this period (see above). However, during the occasional “late” phase (central latency of about 30–35 msec) a facilitation of Ia inhibition was found (Fig 9 A) together with a susceptibility to recurrent depression (Fig 9 B, C). This late response might well have been due to some spinobulbo spinal reflexes (Shimamura 1963; Shimamura and Longstone 1963). It disappeared with spinal transection. These late coFRA effects are not included in Tables I and II.

#### 4. Recurrent depression correlated with facilitation of Ia IPSP

In spinal cats it was often observed that IPSPs evoked by volleys in coFRA which facilitated the Ia IPSPs themselves were depressed by antidromic volleys in motor axon collaterals (Fig 1, 2, 5, 8). This was interpreted as a sign that the coFRA IPSPs at least partly were mediated by the Ia inhibitory interneurons. If this holds true, one should expect a systematic correlation between the facilitation of Ia IPSPs by coFRA and depression of the coFRA IPSPs by antidromic volleys in the recurrent motor axon collaterals. Table II illustrates this correlation for the material of PBSt and Q motoneurons. For individual motoneurons the recurrent depression (ordinate) of IPSP from contralateral high thresh-

old muscle afferents (coH) and from contralateral high threshold cutaneous afferents (coSur) are plotted against the ability by volleys in the same afferents to facilitate Ia IPSPs (abscissa). Each symbol represents one motoneurone and the table gives only the presence or absence of a recurrent depression and of a facilitation of a test Ia IPSP. In several cases it was impossible to decide under which group the result should be classified. Consequently they were omitted from further comparisons. They are, however, presented as a middle group in order to illustrate the frequency at which a decision in any direction was impossible. In the PBSt motoneurones stimulation of the coH revealed a strongly positive correlation between the susceptibility to recurrent depression and ability to facilitate Ia IPSPs. There was neither facilitation of Ia IPSPs nor any depression of the high threshold muscle IPSPs by ventral root volleys in decerebrate cats with a low pontine lesion (unanaesthetized or with chloralose anaesthesia). In the spinal chloralose anaesthetized preparations volleys in these afferents facilitated the Ia IPSPs. The IPSPs evoked by the same afferents were depressed by antidromic volleys.

Stimulation of the coSur nerve demonstrated the same strong positive correlation as found for the contralateral muscle afferents. A facilitation of the Ia IPSPs and a recurrent depression of the high threshold cutaneous IPSPs was only observed in spinal cats anaesthetized with chloralose. A similar relation was also established for the Q motoneurones (right diagram) in which the effects from coH and coSur are put together.

In Table II there are two exceptions to the general rule of the parallelism between susceptibility to recurrent depression and ability to facilitate Ia IPSPs. In one PBSt motoneurone stimulation of the coH nerve did not increase the test Ia IPSP although the coH IPSP itself was depressed by a ventral root stimulation (spinal chloralose preparation). However, a failure to demonstrate spatial facilitation does not necessarily exclude an excitatory convergence on the Ia inhibitory interneurones—the failure can be caused by a small subliminal fringe which would tend to keep the occlusion (also a consequence of convergence) as large as or even larger than the facilitation (see further Fedina and Hultborn 1972, p. 73).

In the other case (in an unanaesthetized low pontine preparation) a coSur volley facilitated the test Ia IPSP in a Q motoneurone while the coSur IPSP itself was not susceptible to recurrent depression. This might first of all indicate some failure of the decerebrate control of the excitatory FRA pathways to the Ia inhibitory interneurones. It is then reasonable to assume that under these conditions the coFRA volley only gives a subthreshold excitation of the Ia inhibitory interneurones, implying that the entire coFRA IPSP recorded is due to activation of interneurones other than those mediating the reciprocal Ia inhibition (cf. p. 73 in Fedina and Hultborn 1972).

Considering the experimental difficulties the obtained correlation between susceptibility to recurrent depression of coFRA IPSPs and the ability by volleys in the same afferents to facilitate test Ia IPSPs must be regarded as remarkably good.

### Discussion

1. *Effects from contralateral H14 on interneurones mediating the reciprocal Ia inhibition.* Bruggenkat and Lundberg (1974) and the present investigation show that a facilitation of transmission in Ia inhibitory pathways to motoneurones can be evoked by volleys in con-

tralateral high threshold muscle afferents (from flexors as well as extensors) high threshold cutaneous afferents and high threshold joint afferents. No qualitative differences could be discerned between the effects on Ia inhibitory transmission from these various afferents. Low threshold afferents on the contrary were always ineffective. This pattern of convergence has led us to conclude that the facilitation of Ia inhibitory transmission is exerted by a contralateral FRA pathway (*cf.* Holmqvist 1961).

Previously Bruggencate *et al.* (1959) and Bruggencate and Lundberg (1974) described this facilitation of Ia inhibitory transmission mainly for knee flexor motoneurons. However in the present investigation (see also Safyants *et al.* 1973) the facilitation of transmission was in addition established in motoneurons supplying some hip and ankle flexors. Furthermore a similar facilitation was revealed in hip (ABSm) and knee extensor motoneurons although normally less spectacular than in the case of flexor motoneurons. It was hard to find any corresponding facilitation of Ia IPSP from coFRA in ankle extensor motoneurons but its presence has recently been reported by Safyants *et al.* (1973) using slightly different preparations. Thus it can be concluded that volleys in contralateral FRA are able to facilitate transmission of Ia inhibition to flexor as well as extensor motoneurons. This conclusion is also supported by direct recording from interneurons supposed to mediate the reciprocal Ia inhibition (Hultborn, Illert and Santini, to be published).

Although Ia inhibitory interneurons inhibiting either flexor or extensor motoneurons can thus be excited by volleys in coFRA, the transmission in the responsible coFRA pathway seems to be very dependent on the type of preparation. Facilitation of Ia IPSPs was regularly found in the spinal preparation after chloralose administration (with the exception of G-S motoneurons) but was rather uncommon in the unanaesthetized spinal state. The less frequent facilitation in the unanesthetized state may depend on a weaker FRA excitation of the interposed interneurons in that preparation or reflect that the excitation is obscured by concomitant inhibitory action. Such a concurrent depression cannot however be caused by an interference at primary afferent level since no PAD in Ia afferents is produced by coFRA volleys in spinal cats (Devanandan *et al.* 1965).

In decerebrate cats with a low pontine lesion coFRA volleys evoke inhibition in flexor and extensor motoneurons (Holmqvist 1961) but coFRA volleys did not facilitate Ia inhibitory transmission in this preparation (unanesthetized or under chloralose anaesthesia, Fig. 7 and 8). On the contrary the coFRA volleys sometimes evoked a depression of Ia IPSPs (Fig. 7) which was caused neither by conductance or potential changes in the motoneurone recorded nor by any recurrent inhibition of the Ia inhibitory interneurons secondary to firing of motoneurons. The reduction of the Ia IPSPs cannot be explained by presynaptic inhibition of transmission from Ia afferents since FRA volleys in decerebrate cats (also after low pontine lesions) do not evoke DRPs (Carpenter *et al.* 1963). Accordingly our findings suggest that the Ia inhibitory interneurons—like the motoneurons (Holmqvist 1961)—receive FRA inhibition in the decerebrate preparations with a low pontine lesion.

From this account it appears that the coFRA pathway which excites Ia inhibitory interneurons seems to be regulated in parallel with the corresponding iFRA pathway (Fedina and Hultborn 1972). Both are thus operating in the chloralose anaesthetized spinal state.



and seem to be obliterated in the low pontine state (with or without chloralose). In the latter preparation activation of coFRA as well as iFRA instead may depress Ia inhibitory transmission presumably by inhibition of the Ia inhibitory interneurons.

Fedina and Hultborn (1972) found that volleys in ipsilateral low threshold cutaneous fibres did facilitate Ia IPSPs also in the decerebrate low pontine state when there was no corresponding facilitation from high threshold muscle and joint afferents. This was taken to indicate the existence of a separate reflex pathway from low threshold cutaneous afferents to motoneurons. In the present study we have been unable to find any evidence for a similar separate crossed cutaneous pathway since all cutaneous effects (which could be evoked only by volleys in high threshold afferents) have conformed with the common coFRA pattern.

## 2. Are inhibitory effects from coFRA mediated by Ia inhibitory interneurons?

The large facilitation of Ia IPSPs sometimes evoked by conditioning volleys in the FRA has raised the question to what extent inhibitory coFRA actions to motoneurons are conveyed by interneurons in the Ia inhibitory pathways. To investigate this problem we have conditioned coFRA IPSPs by antidromic volleys in motor axons known to obliterate transmission via the Ia inhibitory interneurons to the concerned motoneurons. The validity of using recurrent depression of IPSPs as a criteria for their transmission via Ia inhibitory interneurons has been extensively discussed for IPSPs from ipsilateral afferents (Fedina and Hultborn 1972) and more generally for segmental and descending IPSPs (Hultborn 1972).

The present finding of a full convergence of Ia excitation, coFRA excitation and recurrent inhibition leads to the conclusion that if coFRA volleys alone are able to fire these interneurons (as is shown by direct interneuronal recording, Hultborn, Illert and Santini (to be published)) the resulting IPSPs in the target motoneurons would be susceptible to recurrent depression. Assuming that all Ia inhibitory interneurons receive recurrent inhibition (see Hultborn *et al.* 1971b) then the lack of recurrent depression would exclude that the IPSP was transmitted via the Ia inhibitory interneurons. Conversely a conclusion that the recurrently depressed coFRA IPSPs are mediated by the Ia inhibitory interneurons depends on the assumption that interneurons in other inhibitory pathways to the motoneurons are not susceptible to recurrent inhibition. The evidence obtained supports this assumption since measurements from many motoneurons revealed a strong positive correlation between the occurrence of recurrent depression of coFRA IPSPs and facilitation of Ia IPSP (Table II). From these results it is concluded that recurrent inhibition of interneurons mediating crossed inhibition is restricted to the Ia inhibitory interneurons.

Since it is known that Renshaw cells themselves receive recurrent inhibition (Ryall 1970; Ryall, Percy and Polosa 1971) it is necessary to consider the possibility that coFRA IPSPs which are susceptible to recurrent depression in fact may have been mediated via these interneurons. A transmission via Renshaw cells can occur either by direct excitation or secondarily by firing of motoneurons. In order to exclude the latter possibility it is important to control that the afferent volleys used to evoke the test IPSP in the motoneurons do not elicit a discharge in central roots. The possibility that volleys in coFRA may fire Renshaw cells orthodromically is very small since activation of coFRA in lead evokes a powerful inhibition of Renshaw cells (Walsby, Talbot and Kato 1974; Hultborn and Illert, unpublished). If a small

part of the IPSPs nevertheless was to be conveyed by Renshaw cells it is unlikely that even that part would be effectively depressed by conditioning ventral root volleys since the inhibitory interaction between Renshaw cells seems to be weak (Hultborn *et al* 1971c see their Fig. 6).

We thus conclude that recurrent depression of IPSPs from contralateral afferents in motoneurons seems to indicate that—and also roughly to which extent—they are mediated by the Ia inhibitory interneurons.

The extent to which the FRA inhibition is conveyed by the Ia inhibitory interneurons gauged by its sensitivity to recurrent depression is summarized in Table III. This procedure of course assumes that transmission of recurrent inhibition to the Ia inhibitory interneurons is effective in all types of preparation used. As stated in Results it was therefore always controlled that recurrent conditioning volleys effectively depressed Ia IPSPs in the motoneurons investigated.

Although in the spinal state the excitation of extensors and inhibition of flexors from the coFRA is often the dominant action (cf. the pattern in the classic crossed extensor reflex Sherrington 1910) the existence of alternative excitatory and inhibitory pathways from coFRA to both flexor and extensor motoneurons has been demonstrated in this preparation nevertheless (Holmqvist 1961) and intracellular recording from motoneurons usually reveals a mixture of EPSPs and IPSPs (Bruggencate and Lundberg 1974 and present investigation). The regular and sometimes prominent recurrent depression of coFRA IPSPs in flexor motoneurons in the spinal chloralose state indicates that an appreciable part of them are conveyed by Ia inhibitory interneurons (cf. Fig. 1, 2 and 8 D-F). However the coFRA IPSPs were never completely obliterated after conditioning ventral root volleys (sometimes only a marginal depression was seen). This led us to conclude that coFRA IPSPs in flexor motoneurons in the spinal chloralose anaesthetized state besides their mediation by Ia inhibitory interneurons in addition are conveyed by private FRA interneurons (i.e. by interneurons which are not shared with Ia afferents). Using these criteria in extensor motoneurons it seems that only a small fraction of the coFRA inhibition is conveyed by the Ia inhibitory interneurons (cf. Fig. 5) as is also indicated by the fact that the amplitude of coFRA IPSPs may exceed many times the amplitude of the maximal Ia IPSP.

In the spinal unanaesthetized preparation (not included in Table III) that part of the coFRA inhibition which is mediated by the Ia inhibitory interneurons seems to be smaller in flexor as well as in extensor motoneurons. In this state it is however often difficult to judge quantitatively the susceptibility to recurrent depression since the conditioning antidromic volleys themselves frequently evoke a recurrent facilitatory potential and a conductance decrease of the motoneuronal membrane (Wilson and Burgess 1962; Hultborn *et al* 1971c).

In the low pontine state (with or without chloralose) coFRA give rise to inhibition in extensor as well as flexor motoneurons. Since these IPSPs are never susceptible to recurrent depression they should be exclusively mediated by private FRA interneurons (i.e. which are not shared with Ia afferents cf. Fig. 7 and 8 A-C). This was to be expected already from the finding that volleys in coFRA in that type of preparation never facilitate but rather depress Ia inhibitory transmission.

TABLE III The extent to which ipsilateral and contralateral FRA IPSPs in motoneurons are mediated by Ia inhibitory interneurons under different circumstances. The extent to which the FRA IPSPs are transmitted via Ia inhibitory interneurons is based on their susceptibility to recurrent depression (ipsilateral FRA IPSPs: Fedina and Hultborn 1972; contralateral FRA IPSPs: present investigation). See further in the text.

Preparation		Ipsilateral FRA			Contralateral FRA		
		Main effect	Inhibition		Main effect	Inhibition	
			Ia inhib int	Private int		Ia inhib int	Private int
Low pontine (with or without chloralose)	Extensors	Inhib	0	++	Inhib	0	
	Flexors	Inhib	0	++	Inhib	0	+
Spinal, chloralose	Extensors	Inhib	(+)	++	Excit	(+)	
	Flexors	Excit	+	+	Inhib	+	+

Safyants *et al.* (1973) recently reached the conclusion that Ia inhibitory interneurons as a rule do not take an active part in inducing contralateral FRA IPSPs. The preparations in their investigation were not identical to ours, but probably rather comparable to our chloralose anaesthetized spinal state since facilitation of Ia inhibitory transmission was one of their most regular findings. Although the differences in preparation preclude a detailed comparison their conclusion seems to rest on a too indirect experimental approach. It shall be emphasized that our conclusions are not only based on the susceptibility to recurrent depression of coFRA IPSPs but are strongly supported by direct recording from interneurons supposed to mediate Ia inhibition to flexor and extensor motoneurons (Hultborn, Illert and Santini, to be published).

Table III also summarizes the extent to which ipsilateral FRA IPSPs are thought to be mediated by the Ia inhibitory interneurons as judged by their susceptibility to recurrent depression (Fedina and Hultborn 1972).

### 3 The pattern of direct FRA effects on motoneurons in relation to the pattern of FRA facilitation of Ia inhibitory transmission

It has been postulated (Hongo *et al.* 1969; Lundberg 1970) that neuronal pathways which evoke  $\alpha$ -linked movements can achieve a coupling between excitation of agonists and inhibition of antagonists by exerting excitatory action not only in  $\alpha$ - and motoneurons to the agonists but also in the Ia inhibitory interneurons impinging on the motoneurons of the antagonists (see Fig. 10A). Notice that the similar convergence of direct and indirect (from the Ia afferents via the  $\gamma$ -loop) effects governs the excitation of agonist  $\alpha$ -motoneurons and of the interneurons inhibiting the antagonist motoneurons, hence the term  $\alpha$ -linkage in the reciprocal inhibition (Hongo *et al.* 1969). The servo-assistance given by the  $\gamma$ -loop (cf. Matthews 1972, pp. 546-611) will thus support not only the contraction of agonists but to the same degree the relaxation of antagonists.

Activation of ipsilateral as well as contralateral FRA pathways seems often to evoke parallel effects on  $\alpha$ - and motoneurons (cf. Grillner 1969). Since volleys in ipsilateral and contralateral FRA may also evoke excitation of the Ia inhibitory interneurons (Fedina and Hultborn 1972; Safyants *et al.* 1973; Bruggencate and Lundberg 1974 and present study) it seems as if the FRA pathways may represent such a neuronal system with full  $\alpha$ -linkage

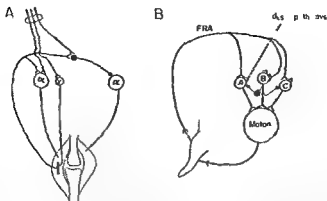


Fig 10 A Schematic representation of a neural pathway with parallel excitatory actions onto  $\alpha$  and  $\gamma$  motoneurons to the agonists and Ia inhibitory interneurons impinging on the motoneurons of antagonists B Diagram showing alternative reflex pathways from the FRA (represented by the neurones A, B and C) with different descending excitatory connexions (dashed lines) The inhibitory interactions between the alternative FRA pathways are illustrated by the inhibition of pathways A and C from pathway B Several of the alternative FRA pathways may be organized to exert a full  $\alpha$ - $\gamma$  linkage as illustrated in the diagram in A (The diagram in B is modified after Fig. 4 in Lundberg 1973) See further in the text

giving a servo assisted control of both excitation and reciprocal inhibition Obviously it is only the reciprocal inhibition mediated by Ia interneurons which is of immediate relevance in the discussion of servo-assisted movements (cf Fig 10 A the private FRA inhibition will be considered later in this section) The dominating excitatory FRA action in the spinal chloralose preparation (see Table III) is exerted onto ipsilateral flexors and contralateral extensors and one would therefore rather expect a reciprocal inhibition (via Ia inhibitory interneurons cf Fig 10 A) mainly in ipsilateral extensor and contralateral flexor motoneurons It is somewhat confusing in relation to this prediction that the most pronounced inhibition via the Ia inhibitory interneurons is seen in flexor motoneurons with either ipsi or contralateral FRA activation (Table III)

The disquieting mismatch between expected and obtained pattern of FRA facilitation of Ia inhibitory transmission may well refer back to more basic questions concerning the organization of the FRA reflex pathways themselves It therefore seems important to review briefly some of the current ideas about the functional organization of these pathways (Lundberg 1973 Lundberg The FRA concept to be published see also in Bruggencate and Lundberg 1974) The main theme includes the following points (cf Fig 10 B) (1) The flexor reflex afferents (FRA) from muscles joints and skin (all with large receptive fields) seem to converge onto common interneurons The activation of the latter from the FRA may thus be essentially lacking space and modality specificity (2) Some of the afferents belonging to FRA are excited during normal limb movements and so do not seem related to any nociceptive reflex function (3) The FRA have several alternative reflex pathways to motoneurons and there is evidence for a mutual inhibitory action between (at least some of) them (4) The interneurons in (at least some of) the various alternative pathways can be activated from descending tracts (5) On the basis of these partly proved assumptions Lundberg (1973) proposed that selective activation (e.g. by descending command signals)



preparation. It seems reasonable to assume that several discrete short latency FRA pathways are organized as to excite  $\alpha$  and  $\gamma$  motoneurons to one group of muscles and the Ia inhibitory interneurons impinging on their antagonists as drawn in Fig 10 A. In view of the complexities their final confirmation must await further experimentation.

After these attempts to reconcile the observations on short latency FRA actions in the spinal cat with the scheme of a full  $\alpha$ - $\gamma$  linkage covering the reciprocal inhibition (Fig 10 A) it deserves to be stressed that many pathways may be organized along completely different principles. There is for example now strong evidence for reciprocal inhibition which is not mediated via the Ia inhibitory interneurons (see e.g. Bruggencate and Lundberg 1974). If the assumption is accepted that inhibition mediated by the Ia inhibitory interneurons subserves  $\alpha$ - $\gamma$  linked movements it might be suggested that reciprocal inhibition mediated via private interneurons represents the reciprocal inhibition in  $\alpha$  movements (Lundberg 1970; Hultborn 1972). However, since the  $\alpha$  motoneurons obviously are fired without the support of the  $\gamma$  loop in  $\alpha$  movements nothing would refute that the same is true for the Ia inhibitory interneurons. It is therefore not possible to exclude *a priori* that the Ia inhibitory interneurons may transmit reciprocal inhibition in  $\alpha$  movements as well as in  $\alpha$ - $\gamma$  linked movements. Be as it may reciprocal inhibition via private interneurons would be characterized by its dissociation from the servo-loop of the agonists (in  $\alpha$  movements of course the servo-loop would not be operating at all) and by its independence of any recurrent control exerted by the active agonist motoneurons.

Furthermore, it shall not be taken for granted that all FRA actions are organized in a simple reciprocal manner: there might well be pathways which increase or decrease excitability of motoneurons to extensors and flexors to different joints in much more complex patterns or perhaps even indiscriminately. It is for example unknown to what extent the possibly no reciprocal low pontine FRA inhibition (see further below) is still present in the spinal state. That pathway might be partly responsible for the FRA inhibition in motoneurons which is not mediated via the Ia inhibitory interneurons (present bilaterally in all species of motoneurons but particularly conspicuous in extensor motoneurons: see Table III).

In contrast to the concurrent activation of several short latency FRA pathways discussed above, it seems possible to obtain a *selective* transmission through a different set of FRA reflex pathways after a *selective* transmission through a different set of FRA reflex pathways after a low injection of 1 DOPA in acute spinal cats (Andén *et al* 1966; Jankowska *et al* 1967). In this state a FRA volley evokes a long latency long lasting excitation of ipsilateral flexor and contralateral extensor  $\alpha$  motoneurons (Andén *et al* 1966) and corresponding  $\gamma$  motoneurons (Grillner, Hongo and Lundberg 1967; Bergmans and Grillner 1969; Grillner 1969). It was recently revealed (Fu, Jankowska and Lundberg 1975) that the concomitant reciprocal inhibition in ipsilateral extensor and contralateral flexor motoneurons is induced mainly, if not exclusively, by Ia inhibitory interneurons. The organization of this late FRA pathway seems thus to be a perfect example of the scheme in Fig 10 A.

In decerebrate cats with a low pontine lesion (Holmqvist and Lundberg 1961; Holmqvist 1961) there seems to be a *selective* transmission through other inhibitory FRA pathways with an organization different from those discussed above. The tonic descending inhibitory control of transmission in all FRA pathways seen in the intercollicularly decerebrate cat (R. M. Eccles and Lundberg 1959; Engberg *et al* 1968 a, b) is selectively released by a low

pontine lesion (Holmqvist and Lundberg 1961; Holmqvist 1961) so that stimulation of FRA then gives rise to a bilateral inhibition in flexor and extensor motoneurons. Since there is no FRA excitation in the low pontine preparation the FRA inhibition in this state, covering all species of motoneurons, can hardly be regarded as reciprocal. As would be expected from that point of view this FRA inhibition is never mediated by the Ia inhibitory interneurons (Table III).

It has to be admitted also that if the "low pontine" FRA inhibition is not "reciprocal" in this state it may nevertheless represent the inhibition which is reciprocal to the FRA excitation seen under other circumstances (e.g. in the acute spinal state in which certainly a part of the FRA inhibition is mediated via private interneurons). That it would assume an entirely differential control of an excitatory pathway and its inhibitory counterpart, it seems more plausible to think of reciprocal inhibition as a "collateral" effect from their excitatory pathway at a rather late stage (for example from the last order interneuron in the excitatory pathway). This would of course not exclude a *pro* its separate control of the reciprocal inhibition since there must be at least one interneuron interposed (the ones inhibiting the antagonist motoneuron). However, with such a scheme it would be rather hard to imagine a full transmission through the reciprocally inhibitory paths while their excitatory counterparts are completely obliterated.

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## Prostaglandin Endoperoxides IX Characterization of Rabbit Aorta Contracting Substance (RCS) from Guinea Pig Lung and Human Platelets

By

JAN SVENSSON MATS HANBERG and BENGT SAMUELSSON

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### Abstract

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Material causing contraction of the isolated rabbit aorta (rabbit aorta contracting substance RCS) was released from guinea pig lung following perfusion with arachidonic acid and from human blood platelets after addition of thrombin to induce aggregation. Prostaglandin endoperoxides (prostaglandins  $G_2$  and  $H_2$ ) were found both in the perfusate of guinea pig lung (1.3 ng/ml) and in the medium collected after platelet aggregation (13-37 ng/ml). The contractile response of the isolated rabbit aorta to the pure prostaglandins  $G_2$  and  $H_2$  was also determined. These data combined with the quantitative analyses of the endoperoxides released from the lungs and platelets showed that only a minor part of the rabbit aorta contracting activity was due to the prostaglandin endoperoxides. The major part of the activity consisted of very unstable material. The half-life of this material was about 30 s at 37 °C whereas at this temperature the prostaglandin endoperoxides had a half-life of about 5 min.

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The release of rabbit aorta contracting substance (RCS) from guinea pig lung following perfusion with bradykinin and slow reacting substance of anaphylaxis (SRS-A) as well as by perfusion of sensitized lungs with antigen was described by Piper and Vane in 1969. Prostaglandins E and F were also detected in the perfusate. RCS could be distinguished from these prostaglandins by its strong activity on the rabbit aorta and by its instability. Vargaftig and Dao later found that also slow reacting substance C (SRS-C) and arachidonic acid caused release of RCS from guinea pig lung. Formation of a rabbit aorta contracting substance during platelet aggregation induced by collagen and arachidonic acid was recently reported by Vargaftig and Zirin.

The finding that aspirin and indomethacin inhibitors of prostaglandin biosynthesis blocked the release of RCS from guinea pig lung indicated a relationship between RCS and the prostaglandins. Gryglewski and Vane suggested that the endoperoxide postulated in the enzymatic conversion of certain polyunsaturated fatty acids into prostaglandins might be identical with RCS. Hamberg and Samuelsson recently isolated a prostaglandin

endoperoxide intermediate which was more active than  $\text{PGE}_2$  on the isolated rabbit aorta. An additional endoperoxide isolated later also had strong activity on the rabbit aorta strip. However the prostaglandin endoperoxides were more stable in aqueous medium than RCS indicating that the latter factor was different from the endoperoxides. Further studies on the nature of rabbit aorta contracting substance released from guinea pig lung and human platelets are described in the present paper.

## Materials

Arachidonic acid purchased from Sigma Chemical Co (purity approx 90%) was purified by silicic acid chromatography and stored in benzene under argon at  $-20^\circ\text{C}$ .

Prostaglandins  $\text{G}_2$  and  $\text{H}_2$  were prepared by incubation of arachidonic acid with sheep vesicular gland microsomes in the presence of *p*-mercuribenzoate (5).

Thrombin (Topostasin® Hoffmann La Roche Co) was used to induce platelet aggregation.

## Methods

### Rabbit aorta strip

Spirally cut strips 30–40 mm long 3–4 mm wide of thoracic aortas from male rabbits weighing 1700–2700 g were suspended and superfused (cf 8) with ml/min of Krebs-Henseleitz bicarbonate medium (6) gassed with 95%  $\text{O}_2$  + 5%  $\text{CO}_2$ . The medium was supplemented with 0.1% glucose and the following antagonists: mepyramine maleate ( $10^{-5}$  g/ml), hyoscine bromide ( $10^{-5}$  g/ml), propranolol hydrochloride ( $10^{-5}$  g/ml), phenoxymethylamine hydrochloride ( $10^{-5}$  g/ml) and methysergide tartrate ( $10^{-5}$  g/ml). The lower end of each strip was attached to a glass rod and the upper end connected to a Grass force displacement transducer FT 03C connected to a Grass model 79C Polygraph. The strips were stretched by a force of 0.5 ponds and allowed to equilibrate for about 1 h. Before each experiment the strips were tested with Hypertensin® (Ciba Geigy AG, Basel, Switzerland) (2.5  $\mu\text{g}/\text{ml}$ ) and with prostaglandins  $\text{G}_2$  and  $\text{H}_2$  (100–200 ng/ml).

### Perfused guinea pig lung

Guinea pig lungs were removed from animals of both sexes weighing 500–600 g. The lungs were cannulated through the pulmonary artery and suspended in a glass chamber (37°C). Krebs medium was perfused into the pulmonary artery (2 ml/min) and the effluent allowed to superfuse the aorta strip. Arachidonic acid (30  $\mu\text{g}$ ) was injected into the cannulated pulmonary artery. In other experiments the lung was perfused with Krebs medium containing 10  $\mu\text{g}/\text{ml}$  of arachidonic acid.

### Homogenization of guinea pig lung

Guinea pig lungs (2–4 g) were minced in 5–10 ml of ice-cold 0.1 M potassium phosphate buffer pH 7.4 and homogenized for 1 min with a Potter-Elvehjem homogenizer. Centrifugation of the homogenate at 8000 g for 15 min gave a supernatant (mainly mitochondrial and soluble fractions) that was used for the incubations.

### Human platelets

Blood from healthy donors who had not taken aspirin for at least 1 week was collected with 7.5% (v/v) of 0.077 M sodium EDTA and centrifuged at 600 g for 15 min. The platelet-rich plasma thus obtained was centrifuged at 650 g for 15 min. The platelet pellet from 1 ml of platelet-rich plasma was suspended in 5 ml of the following medium: 0.15 M NaCl–0.15 M Tris HCl buffer pH 7.4–0.077 M sodium EDTA (90.8  $\mu\text{M}$ ) and subsequently centrifuged. The pellet of washed platelets was prepared in 4 ml of Krebs medium not containing calcium. Platelet aggregation was monitored with a Chronolog aggregometer. Suspensions of 3 ml of washed platelets were stirred at 37°C for 1 min and subsequently treated with 15 U of thrombin. 10 s after the addition of thrombin the medium containing platelet aggregates was filtered with gentle suction and the filtrate was superfused on the aorta strip for assay of RCS activity. For determination of prostaglandin endoperoxides 2 samples (1 ml) of the filtrate were collected and

added to 15 ml of ethanol and to 15 ml of ethanol containing 75 mg of stannous chloride respectively.  $\text{PGF}_2$  was determined in these samples as described below.

#### *Quantitative determination of prostaglandin end peroxides*

Prostaglandin endoperoxides were determined in perfusate from guinea pig lung and in medium obtained after platelet aggregation by multiple ion analysis using [ $3,3,4,4\text{-}^3\text{H}_4$ ]  $\text{PGF}_{2\alpha}$  as internal standard (cf. 5). To 1 ml (platelet medium) or 6 ml (lung perfusate) were added immediately either 15 volumes of ethanol or 15 volumes of ethanol containing 0.5%  $\text{SnCl}_2$ . After 2 min at room temperature 5  $\mu\text{g}$  of deuterated  $\text{PGF}_2$  were added and the solution extracted with diethyl ether. The residue obtained after evaporation was esterified and treated with 50  $\mu\text{l}$  of acetic anhydride and 50  $\mu\text{l}$  of pyridine for 18 h at room temperature. The reagent was evaporated *in vacuo* and the residue chromatographed on a column of 0.1 g of silica acid. Elution was performed with 4 ml of diethyl ether/light petroleum (2/8 v/v) followed by 2 ml of diethyl ether/light petroleum (1/5 v/v). The latter eluate was taken to dryness and subjected to multiple ion analysis. The ions at  $m/e$  314 (unlabeled derivative) and 318 (d-derivative) ( $M-60$  elimination of  $\text{CH}_3\text{COOH}$ ) were used.

## Results

#### *Formation of rabbit aorta contracting substance in perfused guinea pig lung*

Injection of 30  $\mu\text{g}$  of arachidonic acid into the cannulated pulmonary artery of perfused guinea pig lung was followed by a strong contraction of the superfused rabbit aorta strip (Fig. 1). A prolonged contraction occurred following continuous infusion of 10  $\mu\text{g}/\text{ml}$  of arachidonic acid into the lung. No or only small response was seen when arachidonic acid was directly allowed to superfuse the aorta strip. Arachidonic acid (30  $\mu\text{g}$ ) injected following infusion of aspirin (70  $\mu\text{g}/\text{ml}$ ) or 5,8,11,14-eicosatetraynoic acid (30  $\mu\text{g}/\text{ml}$ ) gave only weak contraction of the aorta strip.

The smooth muscle stimulating material was very unstable. By collecting perfusate of guinea pig lung injected with 30  $\mu\text{g}$  of arachidonic acid and incubating the perfusate at 37° for various times prior to test on the aorta strip a series of smooth muscle responses (mm maximum pen deflection) and times of incubation at 37° (seconds) were obtained. These tests were performed in random order to avoid possible sensitizing and potentiating effects on the aorta strip. Plot of the smooth muscle responses vs. time revealed a linear relationship. This was interpreted to mean that two logarithmic processes were involved, i.e. first order decay of the smooth muscle stimulating material and a linear logconcentration-response relationship of the aorta strip. In order to determine the slope of the linear part of the logconcentration-response curve ( $k$ ) perfusates of guinea pig lung injected with arachidonic acid were tested either directly or after rapid dilution with known volumes of Krebs medium. By inserting the  $k$  value so obtained in the equation  $\log\text{concentration} = k \cdot \text{response}$  the concentrations of RCS (arbitrary units) at different times of incubation

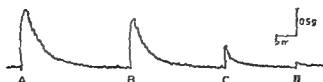


Fig. 1. Contractile responses of isolated rabbit aorta exposed to effluent (10 drops during 15 s) from guinea pig lung injected with 30  $\mu\text{g}$  of arachidonic acid. A: effluent not incubated; B: effluent incubated at 37° for 10 s; C: effluent incubated at 37° for 60 s; II: effluent incubated at 37° for 90 s.

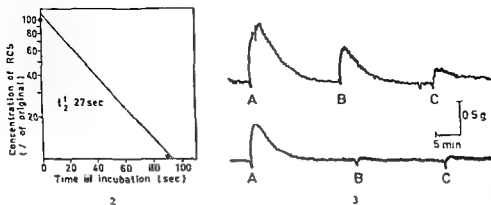


Fig. 2 Decay of rabbit aorta contracting activity in aqueous medium at 37

Fig. 3 Contractile responses of isolated rabbit aorta exposed to medium (10 drops during 15 s) collected after platelet aggregation. Upper curve: A without inhibitor B  $20 \mu\text{g/ml}$  of aspirin C  $40 \mu\text{g/ml}$  of aspirin. Lower curve: A without inhibitor B  $10 \mu\text{g/ml}$  of indomethacin C  $0.7 \mu\text{g/ml}$  of indomethacin.

could be calculated from the smooth muscle responses. The decay of RCS followed first order kinetics and had a half life time equal to  $29 \pm 9$  sec ( $n=4$ ) (Fig. 2).

#### Formation of prostaglandin endoperoxides in perfused guinea pig lung

Arachidonic acid was continuously infused ( $10 \mu\text{g/ml}$ ) into the cannulated pulmonary artery. When RCS appeared in the perfusate as indicated by contraction of the aorta strip the strip was put aside and 2 samples of perfusate were collected. Sample no. 3 (6 ml) (Table I) was collected in a flask containing ethanol and sample no. 4 (6 ml) in a flask containing ethanol +  $0.5 \mu\text{g/ml}$   $\text{SnCl}_2$ . The flasks were switched with short time intervals during collection in order to compensate for possible variations in concentration of the endoperoxides in the perfusate. Samples no. 1 and 2 were also collected in flasks containing ethanol without and with  $\text{SnCl}_2$  respectively. In this case no arachidonic acid was infused into the lung. The results of quantitative determination of  $\text{PGF}_2$  in the samples are given in Table I. As seen before arachidonic acid infusion low and variable amounts of  $\text{PGF}_2$  were found. A higher level was seen after infusion of arachidonic acid (sample no. 3). A pronounced increase was noted in sample no. 4 corresponding to infusion of arachidonic acid and  $\text{SnCl}_2$  reduction. The difference in the amount of  $\text{PGF}_2$  present in samples no. 4 and 3 reflected the level of prostaglandin endoperoxides G or H or both ( $1-3 \text{ ng/ml}$ ).

In order to test whether the measured levels of endoperoxides could be responsible for the aorta stimulating activity of the perfusate the effect on the aorta strip of  $\text{PGG}_2$  and  $\text{PGH}_2$  ( $1-3 \text{ ng/ml}$ ) was tested. No response was obtained.

#### Formation of rabbit aorta contracting substance in homogenates of guinea pig lung

Half a ml of the  $8000 \times g$  supernatant of a homogenate of guinea pig lung was incubated at 37 with  $2-15 \mu\text{g}$  of arachidonic acid for 30 s and subsequently diluted with 4 ml of Krebs medium and allowed to superfuse the aorta strip (10 drops during 15 s). A strong contrac-

TABLE I Release of prostaglandin endoperoxides from guinea pig lung.

Exp. no.	Condition	PGF <sub>13</sub> (ng/ml)
I	1	0.6
	2	1.0
	3	1.5
	4	3.4
II	1	0.1
	2	0.3
	3	1.4
	4	~9
III	1	0
	2	1.4
	3	1.4
	4	4.7

\* 1 No arachidonic acid, no reduction; 2 No arachidonic acid, reduction; 3 Arachidonic acid, no reduction; 4 Arachidonic acid, reduction.

tion was noted. When arachidonic acid was omitted a weak response was recorded. This response was elicited also when the diluted supernatant was kept at 37° for 4–10 min. The response corresponding to arachidonic acid plus supernatant, however, was due to unstable material since it disappeared when the diluted incubation mixture was kept at 17° for 5 min. The half-life time was not determined but appeared to be close to that of RCS formed in guinea pig lung and by platelets.

#### Formation of rabbit aorta contracting substance in platelets

Three ml suspensions of washed human platelets at 37° were treated with 15 U of thrombin. After 30 s the aggregated platelets were removed by filtration and the filtrate was assayed.

TABLE II Release of prostaglandin endoperoxides from platelets

Exp. no.	Condition	Time after aggregation (min)	PGF <sub>13</sub> (ng/ml)
I	1	5	10
	2	5	8
II	1	1.5	1
	2	1.5	5
III	1	1.5	14
	2	1.5	37
	1	10.0	16
	2	10.0	19
	3	10.0	19
IV	1	0	III
	2	0	53
V	1	2.0	18
	2	0	44
VI	1	0	19
	2	~0	41

\* 1 No reduction; 2, Reduction.

(10 drops during 15 s) on the aorta strip. As seen from Fig. 3 a strong contraction was recorded. This was seen only when the platelet medium was tested a short time (0.5–1.5 min) after the aggregation period. Samples kept at 37° for 5 min or more gave no or insignificant response. The half life in the first order decay of RCS from platelets was determined to  $34 \pm 7$  s ( $n=6$ ) using the method described above for RCS from guinea pig lung. When the platelet suspension was preincubated with aspirin or indomethacin formation of RCS was strongly inhibited (Fig. 3). The  $LD_{50}$  dose of aspirin was about 25 g/ml.

#### *Formation of prostaglandin endoperoxides in platelets*

Three ml suspensions of washed human platelets (250 000–500 000 platelets/l) in Krebs medium were stirred at 37° for 2 min and subsequently treated with 15 U of thrombin. Aggregation was allowed to proceed for 30 s. The platelet aggregates were removed by filtration and 1 ml of medium was added to 15 ml of ethanol and to 15 ml of ethanol containing 75 mg  $SnCl_2$ . Table II shows the content of  $PGI_2$  in these samples. The difference between the amounts in the reduced and non reduced samples reflected the level of prostaglandin endoperoxides  $G_2$  and/or H (13–37 ng/ml).  $PGI_2$  and  $PGH_2$  in these concentrations gave very weak response on the aorta strip and could thus not explain the strong rabbit aorta contracting activity of the platelet medium.

### Discussion

In the present work the formation of material(s) with stimulating effect on the rabbit aorta (RCS) was studied in three systems: i.e. guinea pig lung perfused with arachidonic acid, a cell free preparation of guinea pig lung and human platelets treated with thrombin. Formation of RCS in the three systems was inhibited by pretreatment with aspirin, indomethacin and 5,8,11,14-eicosatetraenoic acid. These drugs also inhibit prostaglandin biosynthesis (9,1). The disappearance of rabbit aorta contracting activity produced by guinea pig lung and platelets followed first order kinetics. The half life time of the activity from guinea pig lung was  $29 \pm 9$  s and that from platelets  $34 \pm 7$  s. In a recent study RCS from guinea pig lung was reported to have a half life of 1–2 min (7). Attempts to recover the smooth muscle stimulating activity by rapid extraction of the acidified aqueous phase with diethyl ether (cf. ref. 4 and 5) were unsuccessful.

Prostaglandin endoperoxides ( $PGG_2$  and/or  $PGH_2$ ) were determined in the perfusate from guinea pig lung and in platelet medium recovered after aggregation induced by thrombin. As shown by Table I 1–3 ng/ml of  $PGG_2$  and/or  $PGH_2$  were present in the effluent from guinea pig lung perfused with 10 g/ml of arachidonic acid. Medium collected after platelet aggregation contained 13–37 ng/ml of  $PGG_2$  and/or  $PGH_2$ . Prostaglandin endoperoxides when tested in these concentrations on the rabbit aorta strip gave no or very weak response. This indicated that at least the major part of the rabbit aorta contracting activity formed in guinea pig lung and platelets was due to material which was not identical with the prostaglandin endoperoxides. This was also indicated by the fact that the half life of the endoperoxides in aqueous medium (about 5 min ref. 5) was much longer than that of the rabbit aorta contracting activity. In a separate experiment  $PGG_2$  and  $PGH_2$  were

to perfusate of guinea pig lung and the half life time determined. The values obtained did not differ significantly from those obtained with buffer or water excluding the possibility that an endoperoxide degrading activity was present in the perfusate.

The present study shows that RCS of Pper and Vane is a mixture of at least two components with rabbit aorta contracting activity *i.e.* prostaglandin endoperoxides and an unstable rabbit aorta contracting factor. Further work is needed to clarify the chemical nature of the latter factor.

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## Circulatory and Respiratory Effects Evoked by Hypertonic Ventriculo-Cisternal Perfusion

By

STEFAN MELLANDER AND JAHN HILLMAN

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### Abstract

MELLANDER S and J HILLMAN *Circulatory and respiratory effects evoked by hypertonic ventriculo-cisternal perfusion* Acta physiol scand 1975 94 229-235

The cerebral ventricular system of anesthetized dogs was perfused with synthetic isotonic CSF and for 80 s intervals with hypertonic CSF of various compositions. Hypertonic perfusion evoked centrally mediated marked increases in arterial blood pressure, heart rate, respiratory rate and ventilation and after some delay an excitatory reaction resembling arousal. The responses were coordinated in time with the induced CSF hypertonicity graded in relation to its magnitude and reversible on return to isotonicity. The effects which seemed to be elicited from periventricular structures in the brain stem were more pronounced and consistent when CSF hypertonicity was produced by adding NaCl or Na lactate than monosaccharides to the isotonic CSF solution. Analysis of the cardio-vascular responses indicated that they were caused by increased sympathetic vasoconstrictor and cardiac accelerator fibre activity and by inhibition of vagal discharge to the heart. The described pattern of response much resembles that evoked by physical exercise, a state which might lead to osmolar changes in the brain and CSF of a similar kind to that in the present study as a consequence of the pronounced work induced arterial hyperosmolality. It is suggested that such an osmotic mechanism might constitute a metabolic link in the centrally mediated circulatory and respiratory adjustments in exercise.

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Recent studies from this laboratory have shown that osmotic mechanisms play several important roles in the control of the circulation. Thus tissue hyperosmolality resulting from increased cell metabolism is a factor which contributes significantly to the functional hyperemia in exercising skeletal muscle (Mellander *et al* 1967, Mellander and Lundvall 1971, Lundvall 1972) and in active gland (Lundvall and Holmberg 1974). Tissue hyperosmolality is also mainly responsible for the rapid accumulation of plasma fluid which occurs in skeletal muscle during work (Mellander *et al* 1967, Lundvall 1972). Further, there is an important osmotic regulation of plasma volume in exercise and in hemorrhage effected by pronounced arterial hyperosmolality which leads to fluid withdrawal from tissues to the blood stream (Lundvall *et al* 1972, Jarhult 1973). In exercise this arterial hyperosmolality is caused by an overflow of osmoles from the active muscles (Lundvall *et al* 1972) and in hemorrhage it is caused by glucose release from the liver (Jarhult 1975).



There is also some evidence that arterial hypotension of the magnitude occurring in exercise or bleeding can lead to increased sympathetic nervous activity (e.g. Järhult Hillman and Mellander 1975). This effect might to some extent have been caused by various reflex adjustments from peripheral receptor sites (e.g. Lasser *et al.* 1960 Raizner *et al.* 1973) but, hypothetically it might also have been due to an osmotic effect on the central nervous system. Blood borne hypertonicity would cause a motile dehydration of the brain and the resulting cerebral hypertonicity might influence neuronal activity in central nervous autonomic structures. In the present study an attempt was made to investigate this hypothesis by studying circulatory, respiratory and some other functions during selective cerebral hypertonicity as produced by hypertonic ventriculo-cisternal perfusion in the anesthetized dog. The present paper describes effects observed during short term hypertonic perfusion.

### Methods

Experiments were performed on 16 mongrel dogs, b.wt. 8–10 kg, anesthetized *ex vivo* with  $\alpha$ -chloralose (50 mg/kg) and urethane (100 mg/kg). The animals were provided with a tracheal cannula and breathed spontaneously through a low resistance two-way valve with negligible dead-space. The expiration tube was connected to a spirometer system for continuous recordings of respiratory rate, tidal volume and cumulative pulmonary ventilation. Arterial pulsatile and mean pressures were monitored from a femoral catheter in the right femoral artery and recorded with Statham transducers. Heart rate was recorded with a Grass tachograph using pulse pressure as the trigger. In 3 animals calf blood flow was recorded by inserting an optical drop recorder in the popliteal artery after heparinization. All these parameters were recorded on a Grass polygraph. EEG was registered in two experiments on an Elema recorder using electrode pairs fixed at the surface of the skull.

An 18 gauge needle with a lateral opening 1 mm from its closed tip was implanted in the left lateral ventricle and kept in position with dental cement applied to the small hole drilled in the skull. This cannula was connected to a fine bore polyethylene tubing to a constant infusion apparatus and used for infusion of artificial cerebrospinal fluid (CSF). In two animals a cannula was also implanted in the third ventricle. Five gauge needles were introduced into the cisterna magna through the atlanto-occipital membrane and being used for CSF drainage and the other for intracranial pressure measurements with a Statham transducer. Placement of the cannulas was checked by the occurrence of a spontaneous drainage of CSF and of respiratory CSF oscillations and at the end of the experiment by post mortem analysis of the ventricular system after dissection.

The synthetic CSF was prepared according to Pappenheimer *et al.* (1966) and had the following composition (in %): Na 148, K 3.0, Ca 1.3, Mg 1.6, Cl 131,  $\text{HCO}_3^-$  27, inorganic P 0.5, glucose 3.0. The synthetic CSF made hypertonic (400–1000 mosm/kg  $\text{H}_2\text{O}$ ) by the addition of sodium chloride. The pH of the artificial CSF-solutions was adjusted to 7.40 by bubbling with  $\text{CO}_2$  or  $\text{N}_2$ . The perfusion and temperature was kept at 37°C. Osmolality was determined by thermistor cryoscopic method (Instrument 31 LAS). In Results spread of data is expressed as the standard error of the mean.

### Results

After completion of the preparation the animals rested in the supine right side position. Arterial blood pressure, heart rate, cumulative pulmonary ventilation, respiratory rate, tidal volume and CSF pressure were recorded continuously throughout the experiment. The CSF draining freely through the open needle in the cisterna magna was collected in consecutive 0.2 ml samples for osmolality determinations. Arterial plasma osmolality was determined in intervals. CSF pressure measured at the level of the cisternal outflow cannula, was initially 5–10 mm Hg but fell in the steady-state to 1–2 mm Hg after opening of the

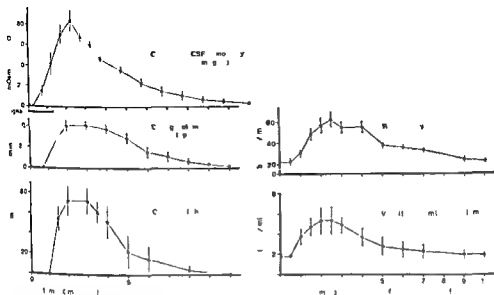


Fig 1 Effects of short term hypertonic ventriculo-cisternal perfusion (signal) on CSF osmolality in the cistern and on mean arterial blood pressure heart rate respiratory rate and cumulative ventilation in anesthetized dogs ( $n=8$ )

drainage. Respiratory oscillations of CSF pressure were always present. Plasma and CSF osmolality did not differ significantly in the control period, the latter osmolality averaging  $310 \pm 3$  mosm/kg H<sub>2</sub>O.

After about 20 min of rest, the infusion of artificial isotonic CSF through the cannula in the left lateral ventricle was started at a constant rate of 0.55 ml/min. CSF drained through the cisternal cannula at the same rate and CSF pressure was therefore unchanged. No significant circulatory, respiratory or other effects were evoked by the isotonic ventriculo-cisternal perfusion.

In this steady state, the isotonic perfusion was changed to a hypertonic perfusion at the same rate (0.55 ml/min) for a period of 80 s, after which the isotonic perfusion was again installed. Fig 1 shows for one group of experiments on 8 dogs the average changes of CSF osmolality as measured in the cisternal fluid and the alterations of mean blood pressure, heart rate and respiration evoked by such short term hypertonic perfusions. In the control period before the hypertonic infusion, mean arterial pressure averaged  $126 \pm 4$  mm Hg, heart rate  $94 \pm 8$  beats/min, respiratory rate  $21 \pm 3$  breaths/min and ventilation  $1.76 \pm 0.12$  l/min. In these experiments, the osmolality of the hypertonic artificial CSF was about 800 mosm/kg H<sub>2</sub>O and the CSF hypertonicity was produced by the addition of sodium salts to the isotonic CSF: Na lactate in 6 experiments and NaCl in 2 expts. The effects were quite similar for both these agents. Zero time on the abscissa refers to the moment the hypertonic CSF reached the left lateral ventricle. It can be seen that the hypertonic infusion (signal) followed by isotonic perfusion caused a transient rise of CSF osmolality which in the cistern reached a peak value after some 2 min of about 80 mosm/kg H<sub>2</sub>O above the control level. The osmolality then gradually returned to the control level in the following 8 min.

It can also be seen that the hypertonic infusion elicited pronounced increases in blood pressure heart rate respiratory rate and cumulative ventilation effects which were quite closely coordinated in time with the CSF hypertonicity. The blood pressure and respiratory responses usually commenced some 15 s before the tachycardia response and during this initial period there were signs of a slight bradycardia (not indicated in the figure). Maximal responses were reached after about 2 min. Mean arterial pressure at this time was increased by about 40 mm Hg, heart rate by about 70 beats/min, respiratory rate by about 40 breaths/min and pulmonary ventilation by about 4 l/min. Tidal volume increased initially but declined again when the tachypnea became marked. All these effects were reversible and were abolished as the time CSF osmolality returned to the control level. CSF pressure was not affected by the hypertonic infusion.

The blood pressure rise during hypertonic perfusion seemed to be caused at least partly by an increase in peripheral resistance mediated by the vasoconstrictor fibres as evidenced in 3 dogs by a concomitant marked constriction of the resistance vessels in the lower leg region, an effect which was absent after regional  $\alpha$  adrenoceptor blockade. The tachycardia seemed to result from increased adrenergic discharge as well as decreased vagal discharge to the heart as evidenced by a much lessened but not abolished response after atropinization (1 mg/kg b.wt. i.v.) or cervical vagotomy. The cardiovascular effects were still present after the respiratory responses were blocked by gallamine (3 mg/kg b.wt. i.v.) in the artificially ventilated dog.

The magnitudes of the blood pressure rise the tachycardia and respiratory responses were graded in relation to the degree of the induced CSF hypertonicity as found during infusions of CSF with both lower and higher tonicity than that in the experiments included in Fig. 1. Clearly discernible effects were noted when cisternal CSF osmolality during the peak (cf. Fig. 1) exceeded the control value by 10–15 mosm/kg  $H_2O$ .

Besides the described circulatory and respiratory effects hypertonic ventriculo-cisternal perfusion led to an excitatory response in the anesthetized dog which in some respects resembled arousal. In some experiments like those included in Fig. 1 the excitation was relatively weak and characterized by slight movements of the head and the extremities, some increase of skeletal muscle tone, semi-opened eyelids and somewhat increased responsiveness to reflex stimuli. The pattern of response which was preceded in time by the circulatory and respiratory effects disappeared as CSF tonicity returned to normal. In some other experiments in particular during higher CSF hypertonicity the excitatory response was much more pronounced. The dog then appeared to be clearly alerted, tended to raise its head and lick its mouth and made distinct movements of the extremities, muscle tone was significantly increased, urination and defecation frequently occurred and the animal reacted clearly to various stimuli such as noise. When these effects developed (usually about 3 min after the start of the hypertonic infusion) there was a secondary reinforcement of the circulatory and especially of the respiratory effects; in one dog ventilation increased to 16 l/min (about 8 times the control value).

Naturally it was considered necessary to administer more anesthetics (chloralose urethane or pentobarbital sodium) in these experiments and this was done routinely as soon as there were any signs of marked arousal. The excitatory response then quickly disappeared.

but a repeated hypertonic ventriculo-cisternal infusion in such deeply anesthetized animals again evoked the described circulatory respiratory and arousal effects. The total amount of anesthetics which the dog received, and survived, in such repetitive infusion experiments sometimes must clearly have exceeded the normally lethal dose.

The circulatory and respiratory responses to hypertonic perfusion could be evoked in dogs in which all surgical wound areas were carefully infiltrated with a local anesthetic agent (Xylocain Astra) prior to the infusion indicating that the effects were not caused by pain sensation during arousal.

A few preliminary experiments showed that during moderate hypertonicity the circulatory and respiratory effects could be elicited in the absence of any significant concomitant changes in the EEG pattern. This finding and the fact that the arousal developed later than the circulatory and respiratory effects suggest that the latter were not initiated by the excitatory response if moderate as in the experiments included in Fig. 1.

Ventriculo-cisternal perfusion with artificial CSF made hypertonic by the addition of glucose or mannitol to the isotonic solution could evoke a similar pattern of response to that described for sodium salts but the effects were more variable and for a given osmolality increase quantitatively less pronounced than those observed with increased sodium concentration.

In two experiments attempts were made to expose only the third and left lateral ventricle to hypertonic perfusion. For this purpose an extra needle was implanted in the third ventricle and was used as the inflow cannula during the perfusion. The cisternal cannula was then closed and the infused fluid was drained through the cannula in the left lateral ventricle. A shift from isotonic to hypertonic perfusion of the third and lateral ventricle in these experiments caused no significant changes of blood pressure, heart rate, respiration, or alertness. The typical pattern of response quickly developed however upon diversion of the hypertonic infusion to the distal sections of the ventricular system which was accomplished by opening the cisternal outflow cannula and closing the drainage from the left lateral ventricle. It thus appears from these preliminary experiments that the structures sensitive to hypertonicity and responsible for the described effects might be localized in periventricular areas of the brain stem.

The pH of cisternal CSF was unaltered or occasionally slightly increased during the hypertonic ventriculo-cisternal perfusion, the latter effect probably being the result of the evoked hyperventilation. Arterial plasma osmolality was not changed by the hypertonic ventriculo-cisternal perfusion.

Intravenous infusion of hypertonic mannitol which in the steady state raised arterial plasma osmolality by about 40 mosm/kg H<sub>2</sub>O (2 expts) led after a delay of 1-2 min to an increase of CSF osmolality in the cisterna magna. After 5 min the osmolality increase in the cistern was about 70% of that in plasma.

### Discussion

This study showed that hypertonic ventriculo-cisternal perfusion in the anesthetized dog evoked centrally mediated increases in arterial blood pressure, heart rate and respiration.

and after some delay an excitatory reaction which resembled arousal. The responses were coordinated in time with the CSF hypertonicity graded in relation to its magnitude and reversible upon return to isotonicity. The effects were more pronounced and consistent when the synthetic CSF perfusate was made hypertonic by the addition of sodium salts than of glucose or mannitol to the isotonic solution. Analysis of the cardiovascular effects indicated that they were caused by increased sympathetic vasoconstrictor and cardiac accelerator fibre activity and by inhibition of vagal discharge to the heart. Results obtained after sympathectomy suggested that humoral agents such as vasopressin did not contribute significantly to the peripheral vasoconstriction.

The effects might at least partly be interpreted to be mediated by a Na<sup>+</sup> sensitive mechanism similar to that described by Andersson *et al.* (1972) for the control of fluid balance and blood pressure. The Na<sup>+</sup> sensitive system for this control was reported to be localized in the vicinity of the third ventricle whereas the effects described in the present study seemed to be evoked mainly from periventricular structures in the brain stem. The exact site of action however remains to be determined.

It appears that the present observations might be taken to indicate a physiologic role of osmotic mechanisms in the central control of the circulation, respiration and perhaps also of alertness. This might for instance apply to the situation of physical exercise in which a pronounced arterial hyperosmolality rapidly develops (Lundvall *et al.* 1972) as a result of water flux into and release of metabolic products from the contracting striated muscle fibres (Lundvall 1972). The arterial hyperosmolality mainly due to raised plasma sodium and lactate concentrations in turn would cause secondary passive dehydration of the brain and intracerebral hypertonicity due to preferential water transfer across the blood brain barrier. CSF tonicity would also increase as shown for instance by the present intravenous hypertonic infusion experiments. In absolute figures the Na<sup>+</sup> and Cl<sup>-</sup> concentrations could seem to be increased to the greatest extent (*cf.* Milhorat *et al.* 1971) a situation thus resembling qualitatively the present hypertonic ventriculo-cisternal perfusions with raised NaCl concentration in the perfusate. The abovementioned hypothesis is strengthened by the fact that the cardiovascular, respiratory and autonomic nervous effects and perhaps also the arousal evoked by hypertonic ventriculo-cisternal perfusion so much resemble the adjustments caused by exercise *per se*. Further Holland *et al.* (1959) found that intra-carotid injections of hypertonic solutions evoked a pattern of response which in several respects resembled the one found in this study. The mentioned osmotic mechanism then might be contributing to the centrally mediated circulatory and respiratory adjustments in work that are believed to be metabolically linked; it may thus be partly responsible for the gradually developing circulatory and respiratory changes during exercise and for the maintenance of such effects in the postexercise period. It is possible that an osmotic mechanism also might contribute to centrally mediated adjustments occurring in other hyperosmolar states e.g. in severe hemorrhage (*cf.* Järhult 1973).

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*Note added in proof.* After this paper was submitted for publication an interesting article by H. Shad and H. Sessler has appeared (Pflug. Arch. 1975 353 107-121) which demonstrates a distinct influence of intracranial osmotic stimuli on renal sympathetic activity.

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## Effect of Insulin on Albumin Production and Incorporation of $^{14}\text{C}$ -leucine into Proteins in Isolated Parenchymal Liver Cells from Normal Rats

By

J. DICH and C. N. GLUUD

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### Abstract

DICH J and C. N. GLUUD *Effect of insulin on albumin production and incorporation of  $^{14}\text{C}$  leucine into proteins in isolated parenchymal liver cells from normal rats* Acta physiol scand 1975 94 236-243

Parenchymal rat liver cells were isolated by a modification of the collagenase method of Quistorff, Bondesen and Grønnet. The cells secreted albumin into the medium and incorporated  $^{14}\text{C}$  leucine both into cell proteins and proteins secreted into the medium. Albumin production measured from the immunological determined increment in the incubation medium was  $1.7 \pm 0.1 \mu\text{g}$  albumin/min per g liver wet wt. This is about 30% of the rate of production in the perfused liver. Addition of insulin ( $10^{-6}$ – $10^{-4}$  M) enhanced albumin production (50–17%) and incorporation of  $^{14}\text{C}$  leucine both into albumin (50–8%) secreted proteins (40–9%) and cell proteins (11–8%). Insulin does not increase the production of albumin by depleting the cells. The effect of insulin on albumin production is compatible with an effect on the rate of synthesis as the specific activity of albumin is unaffected by addition of insulin.

Observations *in vivo* that uncontrolled diabetes is accompanied by loss of body weight, depletion of tissue protein, negative nitrogen balance and lowered concentration of plasma albumin has led to the acceptance of the idea that insulin plays a regulatory role in protein metabolism (Manchester 1970).

Insulin administered *in vitro* to muscle and several other tissues from both normal and diabetic animals enhances the incorporation of labelled amino acids into protein (Manchester 1970), probably indicating an increased rate of protein synthesis. Addition of insulin *in vitro* to livers from diabetic animals also enhances the albumin production and the incorporation of labelled amino acids into protein (Marsh 1961, Penhos and Krahl 1962, 1963). Addition of insulin to livers from normal animals does not increase albumin production (Marsh 1961, John and Miller 1969) and only in some cases increases the incorporation of labelled amino acids into protein (Penhos and Krahl 1962, 1963, Mondon and Mortimore 1967, John and Miller 1969).

In the present investigation isolated parenchymal liver cells from normal rats were used

to study the *in vitro* effect of insulin on albumin production and the incorporation of  $^{14}\text{C}$  leucine into secreted proteins (*i.e.* proteins found in the medium) and cell proteins. These cells have earlier been shown to synthesize albumin (Weigand *et al.* 1971, East, Louis and Hoffenberg 1973) and to be sensitive to insulin (Johnson *et al.* 1972, Zahltén, Stratman and Lardy 1973).

### Materials and Methods

**Animals.** Female Wistar rats weighing approximately 200 g were used. The animals were fed *ad libitum* on a laboratory chow.

**Preparation of liver cells.** Cells were prepared according to the method described by Quistorff *et al.* (1973) with the following modifications. Hyaluronidase was omitted in the perfusion medium and the concentration of collagenase was 0.05%. Further the enzyme medium was perfused through the liver for 25 min the flow rate being 7 ml/min. The yield of cells was 2.5–3.5 ml of tightly packed cells from a 200 g rat.

**Incubation.** The incubation medium consisted of Hanks solution (Hanks and Wallace 1949) containing 15 mM glucose, 10 mM HEPES, amino acids as in rat plasma (Scharff and Wool 1964) and 1% bovine serum albumin. pH was initially 7.4 and decreased at the end of the experiments to 7.2–7.3.

The liver cells were incubated in Warburg flasks at 37°C with atmospheric air as the gas phase. Carbon dioxide was absorbed in KOH in the center wells. Incubation volume was 1.5 ml. The cell concentration was 0.05–0.10 ml of tightly packed cells per ml cell suspension (Hepatocyt 5–10, measured as the usual hematocrit). This corresponded to a protein concentration of 10–20 mg per ml cell suspension (0.5 µCi/L [ $^{14}\text{C}$ ]-leucine and insulin or solvent were added after 15 min of preincubation). As insulin is known to be degraded very quickly in the liver (Field 1972) addition of insulin (or solvent) was repeated after 45 and 75 min of incubation. The relevant parameters were measured in the period from 15 to 120 min.

**Albumin production.** Production of albumin was stopped by chilling on ice. Liver cells were immediately separated from incubation medium by centrifugation. Albumin production was determined from the increment of rat albumin concentration in the medium measured immunologically according to the method of Manini, Cabonara and Hieremans (1965). The extracellular volume in the cell suspension was calculated from the hepatocyt.

In some experiments in which the intracellular amount of albumin was measured the liver cells in the suspension were destroyed by ultrasonication before analysis was performed as described above.

**Incorporation of  $^{14}\text{C}$  leucine to albumin.** The amount of label incorporated into albumin was determined as follows. Rat albumin was added in excess to 0.05 ml supernatant of the cell suspension (prepared as above). All albumin was precipitated with specific anti-rat albumin serum. The precipitate was washed 3 times with physiological saline containing carrier leucine. The final precipitate was dissolved in 500 µl

**TABLE 1** Albumin production (µg per ml cells) and incorporation of  $^{14}\text{C}$  leucine into albumin and secreted proteins (cpm  $10^{-3}$  per ml cells). After 15 min of preincubation  $^{14}\text{C}$  leucine and insulin or solvent were added. The incubation time was 105 min. Figures are given as means  $\pm$  S.E. of 5 expts. each performed in duplicate. The significance of the difference between means was determined by the paired-data *t*-test.

	Control	Insulin ( $10^{-8}$ M)	Insulin ( $10^{-8}$ M)	Insulin ( $10^{-8}$ M)	Insulin ( $10^{-8}$ M)
		Difference	Difference	Difference	Difference
Albumin production	96 $\pm$ 30	443 $\pm$ 41 p < 0.001	152 $\pm$ 43 p < 0.001	344 $\pm$ 64 p < 0.001	143 $\pm$ 35 p < 0.001
Incorporation into albumin	11 $\pm$ 18	34 $\pm$ 54 p < 0.001	8 $\pm$ 45 p < 0.001	56 $\pm$ 4 p < 0.05	47 $\pm$ 17 N.S.
Incorporation into secreted protein	63 $\pm$ 44	874 $\pm$ 68 p < 0.001	747 $\pm$ 54 p < 0.001	694 $\pm$ 47 p < 0.05	69 $\pm$ 5 p < 0.05



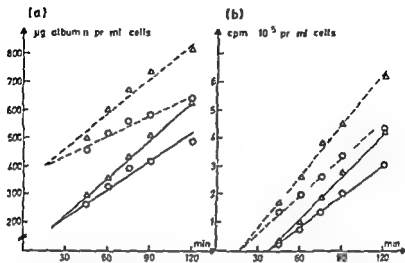


Fig. 1 Time course of albumin production (a) and incorporation of  $^{14}\text{C}$  leucine into albumin (b) in the cell suspension (---) and in the incubation medium (—). Experiment with addition of insulin ( $10^{-4}\text{ M}$ ) is indicated by  $\Delta$  and control by  $\circ$ .  $^{14}\text{C}$  leucine was added at 15 min.

Protocol (New England Nuclear) and counted in a Packard 4000 TriCarb liquid scintillation spectrometer. The incorporated activity was expressed as cpm/ml cells (*i.e.* tightly packed cells).

Control experiments were performed to check if the washing procedure avoids contamination of the albumin precipitate with  $^{14}\text{C}$  leucine. Cell suspensions were preincubated for 15 and 90 min.  $^{14}\text{C}$  leucine was added and the incubations were stopped immediately. Samples were processed as above. In no case the radioactivity in the samples was higher than in the background.

Incorporation of  $^{14}\text{C}$  leucine into secreted proteins and cell proteins: 100  $\mu\text{l}$  supernatant of the cell suspension or 50  $\mu\text{l}$  cell suspension were pipetted on discs of Whatman 3 MM chromatography paper and further processed and counted as described by Mans and Novelli (1961) and Schreiber *et al.* (1971). From the incorporated radioactivity into secreted proteins (*i.e.* proteins found in the medium) and into proteins of the cell suspension the incorporation into cell proteins was calculated. The activity was expressed in cpm/ml cells.

Other analytical methods: Oxygen consumption was measured in a Gilson respirometer and ATP according to Adinolfi (1963).

Chemicals: Insulin was a generous gift from Novo Industri A/S. The hormone was dissolved in dilute hydrochloric acid and neutralized. Final dilution was made with 0.04 M phosphate buffer pH 7.4 containing 1% albumin. Other chemicals were of analytical grade.

## Results

### Albumin production

Addition of insulin increased significantly both the immunologically measured albumin production and the incorporation of  $^{14}\text{C}$  leucine into albumin (Table I). Insulin ( $10^{-4}\text{ M}$ ) increased both parameters 50% (insulin  $10^{-4}\text{ M}$ ) 21% (insulin  $10^{-5}\text{ M}$ ) 16% and 11% respectively and insulin ( $10^{-6}\text{ M}$ ) 17% and 8% respectively. The last mentioned result was not significant. The effect of insulin correlates well for the 2 independent measured parameters ( $r = 0.96$ ,  $P < 0.01$ ).

The increment both in the medium and in the cell suspension of rat albumin concentration proceeded linearly in the period investigated (Fig. 1). In control experiments the im-

TABLE II Incorporation (cpm  $\times 10^{-3}$  per ml cells) of  $^3\text{C}$  leucine at different times into cell proteins in normal parenchymal rat liver cells. Experimental details as in Table I

In- cuba- tion min	Control	Insulin ( $10^{-8}$ M) Dif- ference	Insulin ( $10^{-6}$ M) Dif- ference	Insulin ( $10^{-6}$ M) Dif- ference	Insulin ( $10^{-6}$ M) Dif- ference
15	739 $\pm$ 26	884 $\pm$ 32 145 $\pm$ 7 $p < 0.001$	8.6 $\pm$ 34 87 $\pm$ 9 $p < 0.001$	201 $\pm$ 39 67 $\pm$ 17 $p < 0.05$	799 $\pm$ 37 60 $\pm$ — N S
45	1501 $\pm$ 77	184 $\pm$ 83 323 $\pm$ 36 $p < 0.001$	1668 $\pm$ 86 167 $\pm$ 76 $p < 0.01$	1606 $\pm$ 8.1 105 $\pm$ 8 $p < 0.01$	1548 $\pm$ 85 47 $\pm$ 46 N S
75	1874 $\pm$ 117	2278 $\pm$ 134 404 $\pm$ 6 $p < 0.001$	2015 $\pm$ 1.7 141 $\pm$ 5 $p < 0.01$	1933 $\pm$ 117 59 $\pm$ 6 N S	1883 $\pm$ 109 9 $\pm$ 37 N S
105	200 $\pm$ 146	2539 $\pm$ 169 519 $\pm$ 44 $p < 0.001$	2190 $\pm$ 140 170 $\pm$ 41 $p < 0.01$	2044 $\pm$ 17.4 4 $\pm$ 56 N S	— $\pm$ 1 — $\pm$ 1 N S

munologically measured rate of albumin production was  $2.8 \pm 0.3 \mu\text{g/ml}$  cells per min (Table I). Assuming that 0.6 ml packed cells corresponds to 1 g liver wet wt (Bucher *et al* 1972) this rate corresponds to  $1.7 \pm 0.2 \mu\text{g}$  albumin/g liver wet wt per min which is about 30% of the rate of production in normal perfused rat liver (Hoffenberg, Gordon and Black, 1971; Dich, Hansen and Thieden 1973) but in agreement with earlier observations in isolated liver cells (East, Louis and Hoffenberg 1973). The increment of the incorporation of  $^{14}\text{C}$  leucine into rat albumin followed a similar time-course as the production of albumin determined immunologically (Fig. 1). A time lag period of 15–20 min (transit time) was found before labelled albumin appeared in the medium. This is in agreement with earlier findings in liver slices (Judah and Nicholls 1970) and *in vivo* experiments (Peters and Peters 1972).

The intracellular amount of albumin determined from the difference between the amount of albumin in the cell suspension and in the medium was nearly constant in the period investigated (Fig. 1). Calculated from three independent experiments the intracellular amount of albumin was  $109 \pm 1 \mu\text{g}$  albumin per g liver wet wt which is about 30% of the amount found *in vivo* (Gordon and Humphrey 1961; Peters and Peters 1972). Addition of insulin increased the intracellular amount of albumin with 11%.

#### *Incorporation into secreted proteins and cell proteins*

The incorporation of  $^{14}\text{C}$  leucine into proteins secreted into the medium was enhanced in a similar manner as insulin increased the incorporation into albumin (Table I).

The effect of insulin on the incorporation of  $^{14}\text{C}$  leucine into cell proteins was measured 15, 45, 75 and 105 min after the initial addition of insulin (Table II). Insulin ( $10^{-6}$  and  $10^{-8}$  M) increased the incorporation about 10% and 10% at all times measured. Insulin ( $10^{-6}$  M) gave a significant effect ( $p < 0.05$ ) at 15 and 45 min after addition. The effect of insulin ( $10^{-8}$  M) was not significant.

Incorporation into proteins secreted into the medium showed a similar time-course as the incorporation into albumin (Fig. 1 and 2). Incorporation of  $^{14}\text{C}$  leucine into proteins in the cell suspension started immediately after addition of tracer. The incorporation pro-

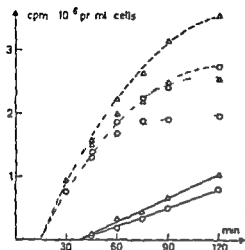


Fig. 2. Time course of incorporation of  $^{14}\text{C}$  leucine into proteins in the cell suspension (—Δ) proteins secreted into the incubation medium (—○) and cell proteins (—◻). Experiment with addition of insulin ( $10^{-6}$  M) is indicated by ◻ and control by ○.  $^{14}\text{C}$  leucine was added at 15 min.

ceeded linearly for 60–75 min. A plateau was reached after about 90 min (Fig. 2). Addition of insulin ( $10^{-6}$  M) resulted in a prolonged linearity and a plateau as in the control experiments was not obtained during the incubation time. The resulting curves for the incorporation into cell proteins have a similar form as the curves for the cell suspension although the duration of the period of linearity was shorter (Fig. 1).

#### *O<sub>2</sub>-consumption and content of ATP*

This was measured to check the viability of the cell preparations.  $\text{O}_2$ -consumption in control experiments was  $1.3 \mu\text{mol O}_2/\text{g liver wet wt per min}$  and declined to  $1.0 \mu\text{mol/g liver wet wt per min}$  at the end of the experiments. The content of ATP at the end of the experiments was  $1.5 \mu\text{mol ATP/g liver wet wt}$ . These results agree with earlier observations (Quistorff *et al.* 1973) and are about 50% of the results obtained in perfused liver (Dich *et al.* 1973).

#### Discussion

It has earlier been demonstrated in perfusion experiments that albumin production (i.e. the amount of albumin liberated into the perfusion medium) is decreased in the diabetic liver (Marsh 1961). Addition of insulin to the perfusion medium increased albumin production (Marsh 1961). A similar effect of insulin on perfused liver from normal animals could not be demonstrated (Marsh 1961; John and Miller 1969). The present investigation shows that addition of insulin to parenchymal rat liver cells from normal rats gives rise to both an increased production of albumin and an increased incorporation of  $^{14}\text{C}$  leucine into albumin.

Further it has earlier been shown that incorporation of labelled amino acids into proteins is decreased in liver slices (Penhos and Krahl 1962), perfused liver (Green and Miller 1960; Penhos and Krahl 1963) and isolated parenchymal liver cells (Ingebretsen *et al.* 1972) from diabetic rats. Addition of insulin increased the incorporation (Penhos and Krahl 1962, 1963).

Attempts to demonstrate a similar effect on livers from normal rats have given equivocal results. With  $^{14}\text{C}$  leucine as tracer no effect of insulin could be demonstrated in perfusion experiments (Penhos and Krah 1963, Mondon and Mortimore 1967). On the contrary Mondon and Mortimore (1967) using  $^{14}\text{C}$  valine and John and Miller (1969) using  $^{14}\text{C}$  lysine found an increased incorporation when insulin was added to the perfusion medium. In the present investigation insulin enhanced the incorporation of  $^{14}\text{C}$  leucine into both cell proteins and secreted proteins.

The reason for this discrepancy between some of the earlier results concerning both albumin production and incorporation of labelled amino acids and the present result is not clear. Meanwhile methodological differences must be considered. Liver slices are not uniform; oxygen diffusion to the cells inside the slices is difficult and the content of ATP is low (Thieden 1968, Grunnet *et al.* 1973). Perfusion experiments are more physiological but the variation between individual livers is considerable if the liver does not act as its own control. In experiments with isolated parenchymal liver cells the results are reproducible, the content of ATP is acceptable, the cells can act as their own control and they represent only one population of cells.

The amount of albumin and the amount of  $^{14}\text{C}$  leucine incorporated into albumin in the intracellular pool was nearly constant both in the control and in the insulin experiments (Fig. 1). This implies that the increased albumin production and incorporation of  $^{14}\text{C}$  leucine into albumin found in the medium could not be due to a depletion of albumin from the cells.

The increased incorporation of  $^{14}\text{C}$  leucine into protein after addition of insulin could theoretically be due to an increased synthesis and/or an increased specific activity of tracer inside the cell. The latter could be caused by an enhanced transport of amino acids into the cell and/or by a decreased degradation of proteins.

Provided that the synthesis of albumin and other proteins in the liver cell use amino acids from the same intracellular pool, an increased transport of amino acids into the cell could not be the only explanation of our results, as insulin did not change the specific activity of albumin. Further, insulin also increased the albumin production determined immunologically (Table I). In contrast to the present findings Miller and Griffin (1972) found that addition of insulin increased the amount of amino acids intracellularly in the perfused rat liver. On the other hand, Schreiber and Schreiber (1972) have shown that isolated parenchymal rat liver cells are freely permeable for leucine and methionine.

Insulin has earlier been reported to decrease the degradation of protein in the perfused liver (Mondon and Mortimore 1967, Mortimore and Mondon 1970). Addition of insulin ( $10^{-6}\text{ M}$ ) increased albumin production by 50% (Table I). If this change should be caused only by a decreased degradation and the considerable rate of protein turnover in the liver ( $t/2 = 16-18\text{ h}$ ) is taken into account, an increase in the specific activity of albumin in the insulin treated cells would have been expected.

The increased albumin production measured immunologically, the increased incorporation of  $^{14}\text{C}$  leucine into albumin and also the unchanged specific activity of albumin are consistent with a direct effect on insulin on the synthesis of albumin. In favour of the regulatory role of insulin on protein synthesis are further more the observations that experi-

mental diabetes results in a loss of rough endoplasmic reticulum and polysomes (Reaven *et al.* 1973) and that administration of insulin to streptozotocin diabetic animals restores the ultrastructure of the liver cell to that seen in normal animal (Pain *et al.* 1974). Insulin also stimulated the formation of polyribosomes in cultures of rat liver cells (Gerschenson *et al.* 1974). The site of action is probably located on the translational level since addition of actinomycin D does not reduce the effect of insulin on the incorporation of labelled amino acids into proteins (Gerschenson, Davidson and Anderson 1974).

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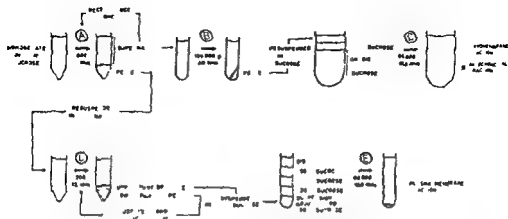


Fig 1 Procedure for the simultaneous isolation of 3 membrane fractions from the rabbit renal medulla. Centrifugations A and D were carried out in an MSE Multix centrifuge; centrifugations B, C and E in a Beckman L 65B ultracentrifuge. The following tubes and rotors were used: A and D 15 ml conical centrifuge tubes; B rotor 50 Ti; C 1.3.5 inch tubes in rotor Sw 27; E 5.9.4 inch tubes in rotor Sw 7. The bands in the gradients were collected with a syringe.

#### Marker enzyme assays

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. 5' nucleotidase (5' ribonucleotide phosphohydrolase, EC 3.1.3.5) was assayed at 37°C with 10 mM adenosine 5' monophosphate (Boehringer Mannheim GmbH, West Germany) in a 0.10 M Tris acetate buffer (pH 7.5) containing 10 mM  $MgCl_2$  (Widnell 1971). Liberated phosphate was determined according to Fiske and Subbarow (1955). Assays were also made with 10 mM adenosine 3' monophosphate or  $\beta$ -glycerophosphate as substrate. For comparison the above procedure was applied to homogenates of rabbit renal medulla, rabbit liver and rat liver. NADH cytochrome c reductase (NADH cytochrome c oxidoreductase, EC 1.6.99.3) activity was assayed by the method of Mahler (1955). Cytochrome c (Sigma Type IV) was used at a concentration of 7  $\mu$ M. Cytochrome c oxidase (Cytochrome c oxygen oxidoreductase, EC 1.9.3.1) activity was determined by the method of Wharton and Tzagoloff (1967). Cytochrome c (Sigma Type IV) as reduced with ascorbate and used at a concentration of 3  $\mu$ M. Acid phosphatase (orthophosphoric monoester phosphohydrolase, pH 5, EC 3.1.3.7) was assayed at 37°C in a 0.05 M acetate buffer (pH 5.0) using 25  $\mu$ M  $\beta$ -glycerophosphate as a substrate. Inorganic phosphate was determined according to Fiske and Subbarow (1955).

#### Assay of prostaglandin synthase

Arachidonic acid was stored frozen as the methyl ester (Applied Science Lab, Pennsylvania, USA). Immediately before use 400–900  $\mu$ g of the ester was hydrolyzed as described elsewhere (Larsson and Ånggård 1974). Tritium labelled arachidonic acid [ $^3H$  6,8,9,11,12,14,15- $H_3$ ] arachidonic acid (NEN Chemicals GmbH, Dreieichenhain, West Germany, specific activity 5–80 Ci/mmol) was added to the non-radioactive substrate before purification on a silica acid column. The radiochemical purity of the tritium labelled arachidonic acid was checked by thin layer chromatography on silica gel impregnated with silver nitrate (system: hexane:diethyl ether:acetic acid 70:30:1 by vol). The arachidonic acid was dissolved in a small volume of ethanol which was then mixed with 0.10 M potassium phosphate buffer (pH 7.4) and added to the incubation medium.

An amount of homogenate or membrane fraction corresponding to 0.15–2 mg protein was incubated in 0.10 M potassium phosphate buffer (pH 7.4) containing 3 mM reduced glutathione, 1 mM EDTA, 0.1 M bovine serum albumin, 16  $\mu$ M (10–80  $\mu$ g/ml) arachidonic acid, 1.5  $\mu$  Ci of [ $^3H$ ] arachidonic acid and 0.05 ml of a supernatant of a boiled 10% homogenate (Samuelsson 1967) of rabbit renal medulla. The total incubation volume was 1.0 ml. Incubations were carried out at 37°C for 0–1 min and terminated by the addition of 9 volumes of absolute alcohol. The samples were then left at +4°C overnight. Double deter-

minations were always performed. Blanks without active enzyme were simultaneously incubated. In the experiments where the distribution of prostaglandin synthetase in different membrane fractions was determined the amount of protein incubated was about 0.5 mg, substrate concentration was 130  $\mu$ M and incubation time 70 min.

After evaporation of the alcohol precipitated samples the aqueous residue was acidified to pH 2-3 with 2*N* formic acid and extracted twice with 3 volumes of diethyl ether. The ether phases were reduced to dryness *in vacuo*. The residue was dissolved in 0.5-1 ml ethyl acetate-benzene (1:1 by vol.) applied on a 0.5 g silicic acid column (Unisil 100-80 mesh, Clarkson Chemical Co., Williamsport, Pennsylvania, USA) and eluted in turn with 30 ml of the following 4 solvents: ethyl acetate-benzene (1:9 by vol.), ethyl acetate-benzene (8:2 by vol.), ethyl acetate and methanol (Hamberg 1969). A 5 ml aliquot of each eluate was counted in a Beckman LS 155 liquid scintillation counter. The scintillator consisted of 4 g of PPO and 0.50 g of POPOP per liter toluene.

The amount of arachidonic acid converted to prostaglandin like substances was calculated from the amount of radioactivity recovered in the 3 last eluates minus blank values and the specific activity of the incubated arachidonic acid. Correction was made for the loss of one tritium atom from C9 of the precursor during the conversion.

Samples of the 4 eluates obtained from the silicic acid column were analyzed by thin layer chromatography on silica gel. The solvent system was chloroform-methanol-acetic acid-water (90:9:1:0.65 by vol.) (Nugteren *et al.* 1966). Prostaglandin  $E_2$  (PGE<sub>2</sub>) and prostaglandin A<sub>2</sub> (PGA<sub>2</sub>) were included as references. The ethyl acetate-benzene (1:9) eluate contained the unconverted [<sup>3</sup>H]arachidonate. The ethyl acetate eluate contained radioactive material mainly co-chromatographing with PGE<sub>2</sub>. The radioactivity in the ethyl acetate-benzene (8:2) and methanol eluates consisted in addition of less polar and more polar compounds respectively.

The inhibitory effect of indomethacin on the conversion of arachidonic acid to prostaglandin like compounds was checked in samples of the homogenate or fraction II incubated as above with a substrate concentration of 130  $\mu$ M and in the presence of indomethacin (Dumex, Copenhagen, Denmark). The prostaglandin synthetase activity was inhibited in both fractions. The inhibition was about 30% at 10<sup>-6</sup> M, 70% at 10<sup>-5</sup> M and 90% at 10<sup>-4</sup> M indomethacin.

## Results

### *Ultrastructure of the isolated membrane fractions*

The ultrastructural appearance of the membrane fractions I, II and III can be compared on the survey electron micrographs in Fig. 2, 3 and 6. The membranes of fractions I and II are shown at high magnification in Fig. 4 and 5.

**Fraction I (plasma membrane fraction)** This fraction consisted of open-ended membrane flakes or vesicles the profiles of which varied in size from 0.2 to 3  $\mu$ m (Fig. 2). When cut at right angles (Fig. 4a and b) they were seen to have a distinct triple layered appearance and a total thickness of 8-9 nm. This corresponds well to the ultrastructure of the plasma membrane of renal cells (Sjostrand 1963) and of plasma membranes isolated from liver cells (Benedetti and Emmelot 1968). Some membranes had an asymmetric triple layered structure (Fig. 4b). This was possibly due to some cytoplasmic material adhering to the inner aspect of the plasma membrane. Five layered structures resembling tight junctions were also observed in this fraction. Membrane fragments resembling distorted mitochondria were seen only very rarely.

**Fraction II (cytomembrane fraction)** This fraction consisted of closed membrane vesicles with a diameter of 0.03-0.2  $\mu$ m (Fig. 3). Both smooth surfaced and ribosome studded membranes were present. When cut at right angles most membranes showed a triple layered structure (Fig. 5a and b) with a thickness of about 6 nm. The triple layered structure was



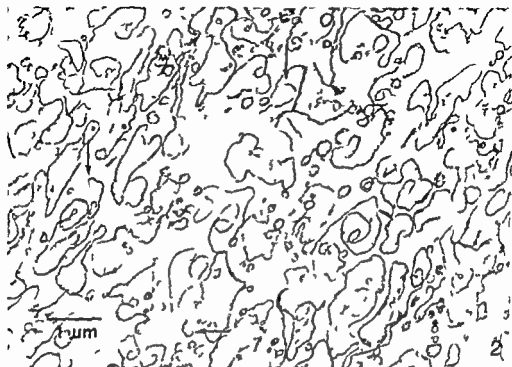


Fig. Survey electron micrograph of fraction I (plasma membrane fraction). It consists mainly of membrane sheets, often with free ends (arrows). Some small vesicles are also present. The size of the membrane profiles is 0.3  $\mu$ m. 13 000.

usually less distinct than that seen in the membranes of fraction I. The presence of some stained material in the light intermediate layer of the membranes often produced a globular pattern. This pattern was also evident in grazing sections, parallel to the membrane surface (Fig. 5b). In contrast to fraction I, only few membranes had a thickness of 8–9 nm. Occasionally membrane limited lysosome like bodies were seen in this fraction.

*Fraction III (mitochondrial fraction)* This fraction consisted almost exclusively of mitochondria (Fig. 6) which often were osmotically affected with a widened outer compartment—intracristal space and a dense matrix. Lysosome like bodies and some rough surfaced membrane vesicles were also present.

#### *Distribution of enzyme markers in the fractions*

The distribution of protein and marker enzymes in the different fractions is seen in Table I and the relative specific activities of the enzymes are shown in Fig. 7.

Cytochrome reductase activity was very low in fraction I, slightly higher in fraction III but increased 2.5 times over the homogenate in fraction II. Cytochrome oxidase activity was low in fractions I and II but increased 3.0 times over the homogenate in fraction III.

It was not possible to determine in the present experiments the activity of 5' nucleotidase in the fractions. Under identical incubation conditions the rate of hydrolysis of adeno-

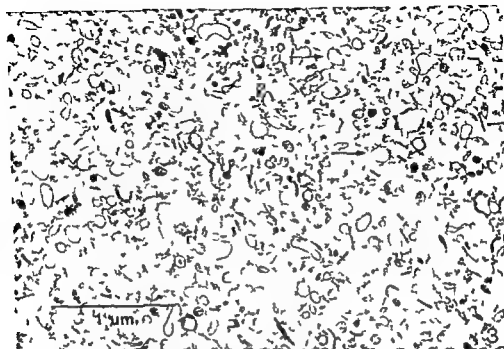


Fig 3 Survey electron micrograph of fraction II (cytomembrane fraction). It consists of small vesicles with a diameter of 0.03–0.3  $\mu\text{m}$ . Both smooth surfaced and rough surfaced (arrows) vesicles are present. Some dense lysosome-like bodies are also seen.  $\times 32,000$ .

sine 3 monophosphate and  $\beta$  glycerophosphate was approximately the same as that of adenosine 5 monophosphate. This was in contrast to rat liver homogenates where the rate of hydrolysis of adenosine 5 monophosphate was about 5 fold that of the other substrates.

TABLE I Distribution of marker enzyme activities in membrane fractions from rabbit renal medulla. For incubation conditions see under METHODS. The values are means  $\pm$  S.E. of 3–5 experiments.

	Homogenate absolute values	Yield in % of homogenate				Recovery	
		I Plasma membrane fraction	II Cyto-membrane fraction	III Mitochondrial fraction	Discard		
Protein	270 $\pm$ 17 mg	0.17 $\pm$ 0.037	4 $\pm$ 0.18	55 $\pm$ 0.30	84 $\pm$ 5	90	7
Cytochrome reductase	13 $\pm$ 6 $\mu\text{mol}$ cytochrome c/min	0.03 $\pm$ 0.018	6.8 $\pm$ 1	9 $\pm$ 0.29	8 $\pm$ 14	7	$\pm$ 1
Cytochrome oxidase	3.7 $\pm$ 0.76 $\mu\text{mol}$ cytochrome c/min	0.058 $\pm$ 0.01	1.8 $\pm$ 0.19	6.9 $\pm$ 1.1	101 $\pm$ 19	110	$\pm$ 0
Acid phosphatase	8.1 $\pm$ 0.34 $\mu\text{mol P}_i$ liberated/min	0.14 $\pm$ 0.051	5.1 $\pm$ 0.0	5.4 $\pm$ 0.30	73 $\pm$ 4	83	$\pm$ 5

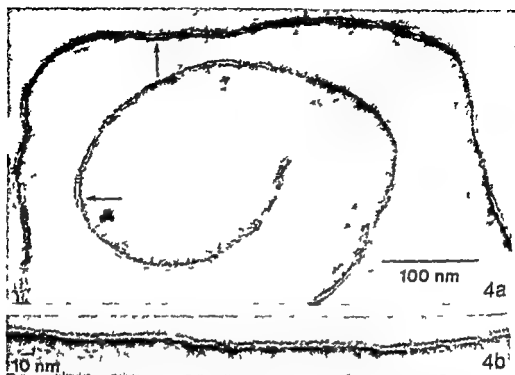


Fig. 4a Membrane sheet in fraction I (plasma membrane fraction). When cut at right angles the membrane is seen to be distinctly triple layered (arrows) and to have a thickness of 8–9 nm. Same magnification as Fig. 5a.  $\times 240\,000$ .

Fig. 4b Membrane in fraction I (plasma membrane fraction). It is distinctly triple layered and asymmetrical. Same magnification as Fig. 5b.  $\times 330\,000$ .

#### *Kinetics of the prostaglandin synthetase assay*

**Effect of substrate concentration.** As shown in Fig. 8 the reaction velocity increased with increasing concentrations of arachidonic acid although the increment in velocity was small for concentrations over  $130\ \mu\text{M}$ . For this reason an arachidonic acid concentration of  $130\ \mu\text{M}$  was used in the incubations.

**Time course.** Under the conditions presently employed (Fig. 9) the reaction proceeded almost linearly for the first 20–30 min. Accordingly an incubation time of 20 min was chosen for the assays. Under these conditions less than 3% of the substrate was consumed in each incubation.

**Proportionality.** The amount of substrate converted to prostaglandin like compounds was proportional to the amount of membrane fraction incubated (Fig. 10). It was found that in order to obtain a sufficient accuracy in the prostaglandin synthetase assay an amount of membrane fraction corresponding to 0.1–0.6 mg protein had to be used for each sample.

#### *Distribution of prostaglandin synthetase in the membrane fractions*

The homogenate of the rabbit renal medulla converted exogenous arachidonic acid to prostaglandin like products at a rate of about  $0.1\ \text{nmol arachidonic acid/min per mg protein}$  (Table

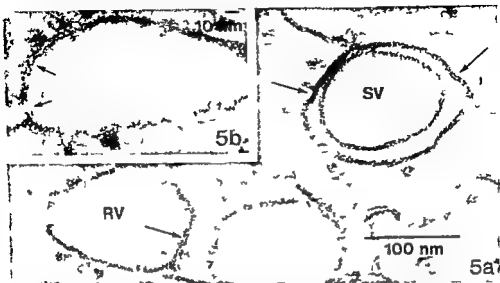


Fig. 5a Smooth-surfaced (SV) and rough-surfaced (RV) vesicles in fraction II (cytomembrane fraction). Both types of membranes are triple layered (arrows) and have a total thickness of 6–7 nm. Compare the thickness of the membrane in Fig. 4a.  $\times 240\,000$

Fig. 5b Rough-surfaced vesicle in fraction II (the cytomembrane fraction). To the right where the membrane is cut at right angles (arrows) it is seen to be triple layered with a total thickness of about 6 nm. Compare the thickness of the membrane in Fig. 4b. In the lower part of the picture where the membrane is cut parallel to its surface a globular pattern is seen.  $\times 330\,000$

II) Prostaglandin synthesis was found in all 3 membrane fractions (Table II). In the plasma membrane (I) and mitochondrial (III) fractions the specific activity of the prostaglandin synthetase was approximately the same as in the homogenate while in the cytomembrane fraction (II) it was 2.4 times that of the homogenate.

TABLE II Distribution of prostaglandin synthetase in membrane fractions from rabbit renal medulla. Samples of the different fractions (about 0.5 mg protein from the homogenate and fractions I, II and about 0.3 mg protein from the discards) were incubated in a 0.10 M phosphate buffer (pH 7.4) at 37°C for 30 min. The concentration of arachidonic acid was  $130\ \mu\text{M}$  and the incubation medium also contained 3 mM reduced glutathione and 1 mM EDTA. The relative specific activity was calculated as (cpm of activity in the fraction) / (cpm of protein in the fraction). Blank values were  $0.55 \pm \text{S.E. } 0.12$  nmol/sample. All values are means  $\pm \text{S.E.}$  of 4 expts.

	Yield in of homogenate	Specific activity (nmol arachidonic acid converted in p.p.m. mg protein)	Relative specific activity
Homogenate	—	$0.098 \pm 0.007$	—
I Plasma membrane fraction	$0.31 \pm 0.054$	$0.11 \pm 0.017$	$1.2 \pm 0.11$
II Cytomembrane fraction	$6.1 \pm 0.46$	$0.1 \pm 0.017$	$4 \pm 0.36$
III Mitochondrial fraction	$5 \pm 0.45$	$0.039 \pm 0.014$	$1.1 \pm 0.4$
Discard	$67 \pm 14$	$0.071 \pm 0.018$	$0.75 \pm 0.15$
Recovery			

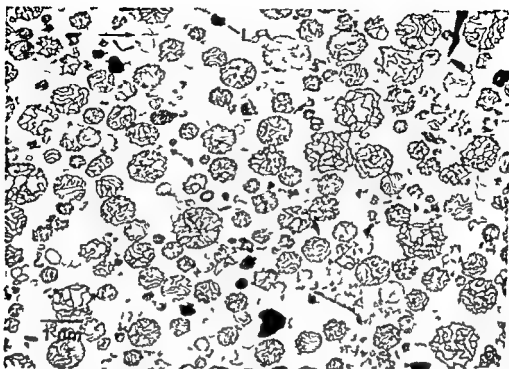


Fig. 6 Survey electron micrograph of fraction III (mitochondrial fraction). It consists predominantly of mitochondria which have a dilated intracristal space and a dense matrix. Some lysosome like bodies (L) and occasional rough surfaced vesicles (arrow) are also present. 10 000.

## Discussion

### *Subcellular origin of the membrane fractions*

*Fraction I* was a plasma membrane fraction as judged by the form and thickness of the membranes. The low relative specific activities of cytochrome reductase and cytochrome oxidase support this interpretation and indicate that contamination by cytoplasmic membranes or mitochondria was minor.

*Fraction II* contained many of the components often considered typical of the microsomal fraction. It has here been referred to as the cytomembrane fraction in order to avoid the often ill defined and misused term 'microsomes' (De Duve 1971). The cytomembrane fraction contained predominantly membranes with a thickness of about 6 nm which often showed a globular substructure (Fig. 5). This corresponds to the ultrastructure of the membranes of the endoplasmic reticulum of intact cells (Sjostrand 1963). The high relative specific activity of cytochrome reductase associated with a low activity of cytochrome oxidase (Fig. 7) supports the view that the membrane vesicles in the cytomembrane fraction derived from the endoplasmic reticulum (Dallner 1963) and that mitochondrial contamination was low. The presence of some 8–9 nm thick membranes indicated that a small amount of plasma membrane fragments were present in this fraction. A quantitative estimate of the amount of plasma membranes in the cytomembrane fraction would be of value but as

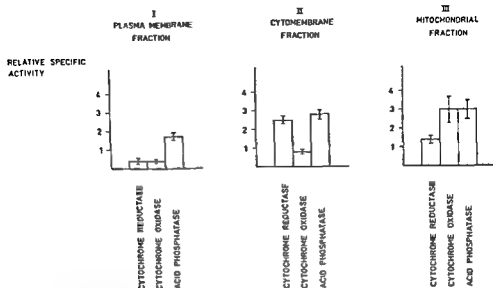


Fig. 7 Relative specific activity of marker enzymes in membrane fractions isolated from rabbit renal medulla. The relative specific activity is calculated as ( $\mu$  of activity in the fraction)/( $\mu$  of protein in the fraction). Brackets indicate  $\pm$  S.E.

discussed by De Pierre and Karnovsky (1973) the use of enzyme markers to detect plasma membranes is problematic. The most widely used plasma membrane marker is 5 nucleotidase (Benedetti and Emmelot 1968; De Pierre and Karnovsky 1973) but the activity of specific 5 nucleotidase (Ahmed and Reis 1958) was very low in the rabbit renal medulla. Furthermore, recent observations by Farquhar *et al.* (1974) indicate that 5 nucleotidase may also be located in cellular components other than the plasma membrane.

The Golgi apparatus is large in some medullary cells (Bohman unpublished). The pro-

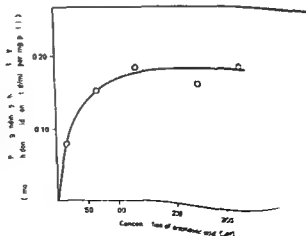


Fig. 8 Effect of different substrate concentrations on the activity of prostaglandin synthetase in the cytomembrane fraction (II) from the rabbit renal medulla. The cytomembrane fraction (0.40 mg protein) was incubated at 37°C for 30 min with different concentrations of arachidonic acid.

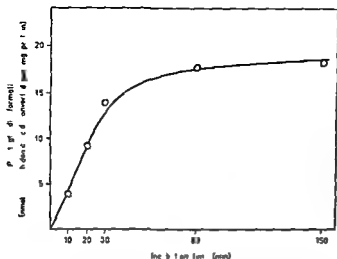


Fig. 9 Time course of prostaglandin synthesis in the cytomembrane fraction (II) from the rabbit renal medulla. The cytomembrane fraction (0.41 mg protein) was incubated at 37°C for varying times. Substrate concentration was 130  $\mu$ M.

ence of Golgi membranes in the isolated fractions was not monitored by enzyme markers but it is possible as judged from the morphology of Golgi membranes and their density in other centrifugation systems (Reid 1967, De Duve 1971) that part of the cytomembrane fraction was derived from this organelle.

Both the ultrastructural and biochemical data show that fraction II also contained some lysosomes.

Fraction III was characterized ultrastructurally and biochemically as a mitochondrial fraction containing additionally some lysosomes and a small amount of membrane fragments from the rough endoplasmic reticulum.

#### Procedure for isolation of membrane fractions

Many methods for isolation of plasma membranes are based on the method of Neville (1960) and involve homogenization of the tissue in water or 1 mM sodium bicarbonate

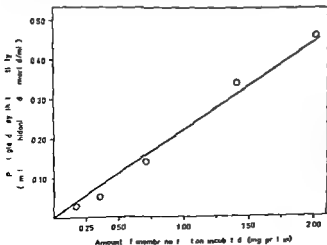


Fig. 10 Proportionality of the prostaglandin synthetase assay. Varying amounts of the cytomembrane fraction were incubated at 37°C for 25 min with a substrate concentration of 130  $\mu$ M.

However the exposure of the various cell components to hypotonic media may prevent the simultaneous isolation of other subcellular fractions (Reid 1967) In this study therefore a gentle homogenization was carried out in 0.3 M sucrose

The total yield of material in the membrane fractions was low This was mainly due to the extensive and tough framework of basement membranes and other extracellular material present in the renal medulla which was only incompletely fragmented during the homogenization A large part of the cellular material was therefore trapped in this framework and sedimented in the low speed pellet

#### *Assay of prostaglandin synthetase*

In order to measure quantitatively the activity of the prostaglandin synthesizing enzyme complex of the rabbit renal medulla we used a radioactive assay with [ $^3\text{H}$ ]-labelled precursor Since  $\text{PGE}_2$ ,  $\text{PGF}_2$  and  $\text{PGA}_2$  are known to be the major prostaglandins of the renal medulla (Daniels *et al* 1967, Lee *et al* 1967) arachidonic acid was used as a precursor (Bergstrom *et al* 1968)

A proportion of the exogenous arachidonic acid was converted to prostaglandin like compounds in the absence of active enzyme in the incubation medium (Nugteren *et al* 1967) The amount of prostaglandin like substances formed non-enzymatically was independent of the incubation time and they appeared to arise mainly during the subsequent extraction and separation procedure The non-enzymatic conversion gave rise to high blank values and thus limited the sensitivity of the method

The somewhat low recovery of prostaglandin synthetase (76%) raises the question whether some physical or chemical inactivation of the enzyme system occurred during the isolation of the membrane fractions However the low recovery may at least in part be attributed to the difficulties involved in the assay of the pooled discard This fraction was very dilute and only about 0.3 mg of protein could be incubated under the conditions employed The determination of such a small amount of enzyme was on the limit of the sensitivity of the method Lands *et al* (1971) reported on a self-catalyzed inactivation of soybean lipoxigenase which may also occur in the initial step of prostaglandin synthesis but it is difficult to judge whether this has had any influence on the present results

Pace Asciak and Wolfe (1970) studying prostaglandin synthesis in rat stomach homogenates found that a considerable dilution of the added substrate by endogenous arachidonic acid took place during incubation and that prostaglandins were formed from both substrates We therefore determined the amount of endogenous arachidonic acid in the different subcellular fractions by gas liquid chromatography (Ånggård *et al* 1972) The total amount of arachidonic acid was in the plasma membrane fraction 110  $\mu\text{g}$  in the cytomembrane fraction 43  $\mu\text{g}$  and in the mitochondrial fraction 31  $\mu\text{g}$  per mg protein We have previously shown that the main part was in phospholipids and only about 1/10 in non polar lipids (Ånggård *et al* 1972) Since each incubation included about 0.5 mg protein the dilution of the 40  $\mu\text{g}$  exogenous arachidonic acid by endogenous precursor was relatively small Furthermore the dependence of the prostaglandin synthetase activity upon exogenous substrate concentration (Fig. 8) did not suggest any major dilution of the added substrate It is believed that at least in some tissues the endogenous esterified fatty acid first has to



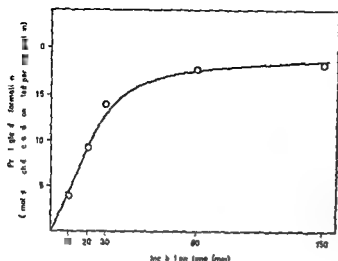


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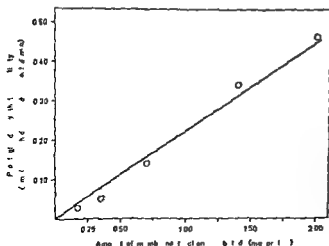


Fig 10 Proportionality of the prostaglandin synthetase assay. Varying amounts of the cytomembrane fraction were incubated at 37°C for 25 min with a substrate concentration of 130  $\mu$ M.

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## Consequences of Myocardial Structural Adaptation on Left Ventricular Compliance and the Frank-Starling Relationship in Spontaneously Hypertensive Rats

By

MARGARETA HALLBACK OLLE ISAKSSON and EDDY NORESSON

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### Abstract

HALLBACK M O ISAKSSON and E NORESSON *Consequences of myocardial structural adaptation on left ventricular compliance and the Frank-Starling relationship in spontaneously hypertensive rats* Acta physiol scand 1975 94 259-270

The Frank-Starling relationship of hearts from adult spontaneously hypertensive rats (SHR Okamoto 1969) representing the established phase of hypertension and of young SHR representing the initial phase of hypertension was investigated by using the isolated working heart preparation. In the normal diastolic pressure range (5 to 10 cm H<sub>2</sub>O) the left ventricle of both SHR groups displayed significantly reduced stroke volumes compared with hearts of normotensive controls (NCR) the degree of reduction being proportional to the left ventricular hypertrophy. This is suggested to be due to a reduced left ventricular diastolic compliance in SHR as indicated by direct measurements of ventricular wall thickness and end-diastolic volumes in arrested hearts exposed to different end-diastolic filling pressures. Such a progressive shift of the Frank-Starling relationship to the right with duration of hypertension could in combination with the gradual development of "structural autoregulation" of the precapillary resistance vessels constitute dominating factors in shifting the hemodynamic situation in labile hypertension into that characterizing the established or "fixed" state of hypertension.

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Left ventricular hypertrophy is a generalized finding in established hypertension and its initiation is proposed to be due to the increased tension development per beat when the left ventricle contracts against an enhanced aortic pressure (cf Bader 1964). In the spontaneously hypertensive rat (SHR Okamoto 1969) being one of the best animal analogues for essential hypertension in man left ventricular hypertrophy seems to occur from quite early age in an extent proportional to the increased arterial pressure (Farmer *et al* 1974). Such a structural adaptation to an increased pressure load has also been demonstrated in the resistance vessels in SHR and in patients with essential hypertension (Folkow *et al* 1973 1974). The hemodynamic consequences of such a "structural autoregulation" will be an increased resistance without necessitating any increased degree of smooth muscle shortening due to the presence of an increased wall/lumen ratio in the resistance vessels.

In labile hypertension evidently preceding "established" essential hypertension (Eich *et al* 1966, Frohlich *et al* 1969) as well as in the initial phase of SHR hypertension (Pfeffer and Frohlich 1973) cardiac output (CO) is increased and total peripheral resistance (TPR) normal (Julius and Schork 1971). The hemodynamic pattern of established essential hypertension and fixed diastolic SHR hypertension is however characterized by a largely normal CO but a clearly increased TPR though with a tendency of a relative increase in muscle blood supply (cf Pickering 1968).

Particularly early phases of essential hypertension often exhibit signs of enhanced adrenergic sympathetic activity (cf Julius and Esler 1975) but if this were generalized and uniform it would tend to increase both CO and TPR. Since however, neither labile nor established essential hypertension show a hemodynamic pattern of exactly this character some compensatory or autoregulatory adjustments seem to take place or/and the increased sympathetic activity is differentiated in nature. The structural adaptation of the resistance vessels, large arteries and left ventricle must be considered as perhaps the most important secondary adjustment since it appears to occur so early in hypertension that it becomes of importance both for the initiation and maintenance of the hypertensive state (Folkow *et al* 1973). Concerning the possible involvement of a differentiated sympathetic influence in hypertension the cardiovascular adjustments both in labile hypertension and initial SHR hypertension display a similar hemodynamic pattern as that elicited by arousal in normotensive individuals when the defence reaction is initiated. Further this reaction indicating a differentiated sympathetic drive on the cardiovascular system is more easily elicited in SHR than in normal control rats (NCR) in a way which reveals an inherent central hyperreactivity to environmental stimuli in SHR (Hallback and Folkow 1974). In the long run such intensified blood pressure increases as initiated by the defence reaction tend to raise the average pressure load on heart and precapillary vessels thereby gradually initiating adaptive structural changes within the high pressure cardiovascular sections characterizing manifest hypertension. Thus differentiated and in principle intermittent rather than tonic and generalized increases in sympathetic activity seem to be involved in the initiation of SHR hypertension.

Also adult SHR with manifest hypertension display more vivid defence reactions than age matched controls though the resting steady state is no longer characterized by an increased CO as tends to be the case in earlier phases. The present study was therefore undertaken to investigate if an altered Frank-Starling relationship of the hypertrophied left heart could at least in part be responsible for the normalization of CO in the established phase of SHR hypertension. For this purpose isolated beating hearts from young and adult SHR and matched NCR were perfused at varying end-diastolic filling pressures in an antegrade perfusion system. In addition the pressure-volume characteristics of the left ventricle were explored in arrested SHR-NCR hearts.

### Methods

**Animal.** Two different age groups of male SHR are used in the present study. Those referred to as "young" SHR were 6 week old and weighed approximately 100 g, while those referred to as "adult" SHR were 30 week old and weighed approximately 150 g. Age matched NCR were used as controls.

**Heart perfusion technique** Before starting the perfusion, arterial pressure was measured in the tail artery during awake conditions (Weiss 1974). During ether anesthesia the hearts were excised and transferred in ice-cold saline which stopped the contractions within seconds. The aorta was connected to the perfusion apparatus using two different sizes of stainless steel cannulas since the rats belonged to two different weight groups. The outer diameter of these cannulated steel cannulas was such that the aortas had to be widened slightly during mounting the heart. The hearts were then perfused via the aorta with non-recirculating Krebs bicarbonate buffer at 37°C (Krebs and Henseleit 1933) from a reservoir 70 cm above the heart, whereupon the hearts immediately started to beat. This preperfusion lasted for 5 min during which the left atrium was mounted on another cannula (outer diameter 3 mm) leading to an atrial bubble trap the level of which determined the left ventricular filling pressure (for details see Isaksson 1973). Both the aortic and atrial cannulas were connected to Tygon tubes with an inner diameter of 3 mm. Following the initial 5 min perfusion period the heart was perfused via the left atrium in an anterograde fashion as a working heart preparation at 37°C (Isaksson 1973). To add some distensibility to the rigid aortic tubings an artificial Windkessel in the form of a side-arm filled with 5 ml of air was always connected to the tube just above the aortic steel cannula. The perfusate entering the left atrium was pumped by the left ventricle to the top of an oxygenating chamber against a hydrostatic pressure of 70 cm H<sub>2</sub>O thus allowing the heart to perform a substantial mechanical work in vitro. It is important to point out that with this hydrostatic pressure level above the heart the left ventricular afterload was nevertheless varied to some extent along with alterations in cardiac output and thus the cardiac work performance was reflected both by the output and the consequently modified afterload. Cardiac work was changed by varying left atrial filling pressure accomplished by changing the level between the atrium and the bubble trap.

The recirculating volume (50 ml in all experiments) was continuously gassed with 95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub>. Krebs-Henseleit bicarbonate buffer was used as perfusion medium in all experiments. Final concentrations of the electrolytes in the buffer were (mM): NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2 and NaHCO<sub>3</sub> 25. The buffer contained glucose at a concentration of 10 mM in all experiments. Coronary flow was measured by collecting the fluid dripping from the heart and aortic flow was measured by unclamping a side arm to the aortic tube at the top of the perfusion apparatus close to the oxygenating chamber. The pressure head above the heart was recorded via the Windkessel side tube which was connected to a pressure transducer and a Sanborn recorder model 16-1300 S. After perfusion the left ventricle was cut off, placed in a vacuum oven at a temperature of 100°C for 3 days whereafter they were weighed.

**Determination of left ventricular compliance** Beating hearts of 7 adult SHR and 10 matched NCR anesthetized with ether were excised and dropped into a beaker containing ice-chilled Krebs bicarbonate buffer. A cannula with an outer diameter of 4.5 mm was rapidly inserted into the left ventricle via the left atrium fitting smoothly into the AV orifice. A narrow rim at the cannula edge made it possible to withdraw the cannula so that its end was placed just at the mural section after ligation at the AV border. Thereafter a bulbous polyglass rod was placed in the aortic root so that it obstructed the aorta and the coronary orifices without encroaching upon the ventricular cavity thus preventing fluid leakage into the aorta and the coronary vessels. Care was taken to avoid air bubbles entering the left ventricle during these cannulation procedures. The cannulated heart was then placed onto a moistened ice-cold gauze and the right ventricle was cautiously cut away from the left ventricular preparation. The wide AV cannula was then connected to a stainless steel Y-shaped tube containing ice-chilled Krebs bicarbonate buffer, one end being fixed to a force displacement transducer for continuous recording of changes in weight of the left ventricular preparation while the sidetube was connected via Tygon tube to a reservoir of oxygenated ice-cold Krebs bicarbonate buffer. By raising or lowering this reservoir known changes in intraventricular pressure could be induced as measured via a thin plastic tube inserted into the AV cannula into the ventricle and connected to a Statham pressure transducer writing on a Grass polygraph. To make sure that the left ventricular preparations had not passed to rigor it was first checked that they responded with contractions to electrical stimulation and when they had regained complete relaxation a standardized and rapid pressure-volume recording was performed at constant temperature condition. It was repeatedly checked in control preparations that the ventricles were still able to respond with well-coordinated contractions upon electrical stimulation also at the time when the recording of the pressure-volume relationships had been completed. Efforts to obtain a true estimation of the intraventricular diastolic volume at the filling pressure of 11 cm H<sub>2</sub>O throughout the series were in this state of stress immediately frozen in liquid nitrogen.

The frozen ventricles were weighed then cut open carefully blotted and weighed again. To simplify

the situation the density of the buffer and the left ventricular wall were considered to be equal and set at 1. The difference between the weight of the frozen ventricle with the buffer content and the weight of the thawed empty ventricle then gives the intra ventricular volume (weight) at the moment of freezing, i.e. when distended at a pressure of 11 cm H<sub>2</sub>O. From this value the exact volumes at the different pressures could be obtained from the recorded changes in weight then giving the pressure-volume curve.

Statistical analyses were performed according to group comparison *t* test.

## Results

Mean arterial pressure was  $164 \pm 5$  mm Hg in the awake adult SHR ( $n=13$ ) representing the established phase of hypertension and  $135 \pm 6$  mm Hg in the young SHR ( $n=10$ ) representing initial labile phases of hypertension. Mean arterial pressures in the corresponding matched NCR were  $119 \pm 4$  ( $n=15$ ) and  $110 \pm 3$  ( $n=8$ ) mm Hg respectively (Table I and II). It is also seen from Table I that the ratio between left ventricular dry weight and body weight (mg/g) was approximately 80 per cent greater in adult SHR compared to age matched controls whereas the corresponding ratio for the young SHR was only some 40 per cent greater compared with their matched controls. These measurements thus demonstrate an increasing degree of left ventricular hypertrophy with extent and duration of hypertension. Table I also shows that the intrinsic heart rate which did not change during the course of the experiment tended to be somewhat though not significantly lower in the two SHR groups than in the corresponding NCR.

*1 Frank-Starling relationship of adult SHR* Fig. 1 illustrates the influence of increasing diastolic filling pressure (DFP) on stroke volume (left part) and on stroke work (i.e. the product of stroke volume and the difference between mean aortic pressure and left diastolic filling pressure right part) of spontaneously beating hearts from adult SHR and matched

TABLE I Left ventricular dry weight/body weight, heart rate, stroke work and stroke volume at diastolic filling pressures (DFP) of 8 and 15 cm H<sub>2</sub>O of isolated hearts from young SHR, adult SHR and matched NCR. The ratio between SHR and NCR for each parameter and the degree of significance are illustrated. Blood pressure for the respective groups are also given. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Values presented are means  $\pm$  S.E.

	Young SHR (6 weeks)			Old SHR (30 weeks)		
	SHR	NCR	SHR/NCR	SHR	NCR	SHR/NCR
Mean arterial blood pressure mm Hg	$135 \pm 6$	$110 \pm 3$	1.2	$164 \pm 5$	$119 \pm 4$	1.4
Left ventricular dry weight/body weight mg/g	$0.66 \pm 0.07$	$0.48 \pm 0.01$	1.4	$0.68 \pm 0.04$	$0.37 \pm 0.01$	1.8
Heart rate beats/min	$281 \pm 11$	$308 \pm 16$	0.9	$63 \pm 11$	$80 \pm 16$	0.9
Stroke volume $\mu$ l						
DFP = 8	$82 \pm 5$	$94 \pm 4$	0.9	$130 \pm 14$	$174 \pm 10$	0.8
DFP = 15	$131 \pm 3$	$127 \pm 6$	1.0	$249 \pm 21$	$248 \pm 10$	1.0
Stroke work g m $10^3$						
DFP = 8	$65 \pm 5$	$67 \pm 4$	1.0	$127 \pm 15$	$193 \pm 11$	0.7
DFP = 15	$107 \pm 4$	$86 \pm 8$	1.3	$265 \pm 24$	$273 \pm 4$	1.0

## SHR-NCR 30 WEEKS OLD

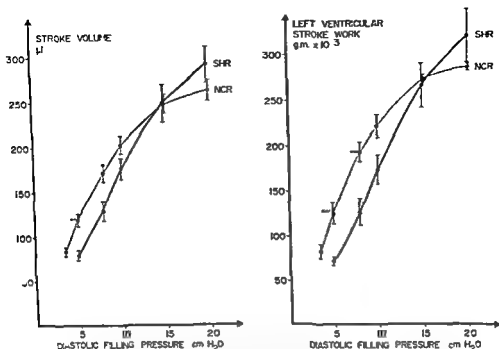


Fig 1 Effect of increased diastolic filling pressure on stroke volume (left part) and stroke work (right part) of SHR aged 30 weeks *ie* old SHR and age matched controls (NCR). Vertical bars represent  $\pm$  S.E.  $-p < 0.05$   $-p < 0.01$   $-p < 0.001$

controls. A comparison of the right and left parts reveals that changes in stroke work fairly closely reflect changes in stroke volume. In the lower range of diastolic filling pressures the SHR hearts produced significantly less stroke work than the NCR ones for a given filling pressure. This difference becomes less pronounced at higher diastolic filling pressures since the Starling curve representing the NCR hearts tend to level off at a lower DFP than that of the SHR hearts. At the highest DFP tested the stroke work of SHR actually exceeded that of the NCR ones. Fig 1 thus demonstrates that in the lower physiological range of diastolic filling pressures a higher filling pressure is required for the SHR left ventricles to produce the same stroke work or stroke volume as the NCR left ventricles.

At all levels of DFP the coronary flow/unit dry weight of the SHR hearts was lower than that of the control hearts.

**II Diastolic left ventricular compliance of adult SHR** In order to further evaluate the background of the altered Frank-Starling relationship of the SHR left ventricles the compliance of left ventricles taken from 7 adult SHR and 10 age matched NCR were investigated as described in Methods. The percentual change in left ventricular volume ( $\Delta V/V$ ) per change in diastolic filling pressure (P) *ie*  $\Delta V/V \cdot \Delta P$  was taken as an index of left ventricular compliance. With the present technique there was no significant difference



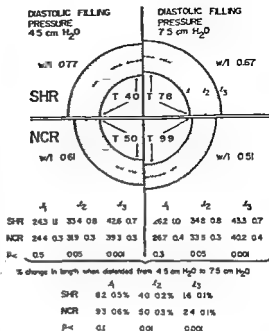
TABLE II Blood pressure left ventricular wet weight, as well as end-diastolic volume and tangential tension per unit tissue mass expressed as relative units  $\left(T = \frac{\text{mm Hg} \times \text{mm}}{\text{mm}}\right)$  at increasing diastolic filling pressures in adult SHR and corresponding NCR Values presented are means  $\pm$  SE Levels of significance are given

	Blood pressure mm Hg	Left ventricular wet weight mg	P=4.5 cm H <sub>2</sub> O		P=7.5 cm H <sub>2</sub> O		P=11 cm H <sub>2</sub> O	
			End diastolic volume ( $\mu$ l)	Tension	End diastolic volume ( $\mu$ l)	Tension	End diastolic volume ( $\mu$ l)	Tension
SHR n=7	164 $\pm$ 5	1063 $\pm$ 43	249 $\pm$ 25	4.0 $\pm$ 0.3	315 $\pm$ 30	7.8 $\pm$ 0.5	377 $\pm$ 30	13.1 $\pm$ 0.6
NCR n=10	119 $\pm$ 4	782 $\pm$ 23	246 $\pm$ 10	5.0 $\pm$ 0.2	322 $\pm$ 14	9.9 $\pm$ 0.4	394 $\pm$ 16	17.1 $\pm$ 0.4
Significance p<	0.001	0.001	0.5	0.01	0.4	0.001	0.3	0.001

between SHR and NCR concerning mean left ventricular volumes at a given end-diastolic pressure over the range of 4.5–11 cm H<sub>2</sub>O (see Table I). However the ventricular compliance as calculated for each individual heart tended to be somewhat lower in SHR than in NCR (6.2 $\pm$ 0.5 per cent versus 7.1 $\pm$ 0.5 per cent  $p < 0.1$ ) when the filling pressure was raised from 4.5 cm to 7.5 cm H<sub>2</sub>O.

The presence of an increased wall thickness in SHR will however in another important way influence left ventricular distention which becomes evident by calculations of average changes in muscle fibre length along the inner mean and outer circumferences of the left ventricles in SHR and NCR. These changes in muscle fibre length along the mentioned circumferences were calculated at DFP of 4.5 and 7.5 cm H<sub>2</sub>O and are presented as means  $\pm$  SE in Fig. 2. The calculations were performed in the following way. From the pressure-volume relationship the end diastolic volumes were obtained at the pressures of 4.5 and 7.5 cm H<sub>2</sub>O. Since the wet weight of the ventricle was determined it was possible to calculate the total ventricular volume at the different pressure levels assuming the ventricle to be a sphere and the density of the buffer and the ventricular wall to be one. Since the total ventricular volume i.e. intraventricular volume plus the volume of the wall was estimated the internal and external radii could be calculated. For each ventricle it was then possible to deduce the wall/lumen ratio and also the mean radius corresponding to the midline of the ventricular wall. From the values of the inner mean and outer radii the respective circumferences could be calculated. Fig. 2 demonstrates the changes in left ventricular geometry of SHR hearts (upper circle) and NCR hearts (lower circle) when DFP was increased from 4.5 cm H<sub>2</sub>O to 7.5 cm H<sub>2</sub>O. As a consequence of the increased wall/lumen ratio in the hypertrophied SHR ventricles the percentual increase in average or mean muscle fibre length (1 in Fig. 2) for this rise of intraventricular pressure is about 20 per cent smaller in SHR than in NCR ( $p = 0.01$ ). When the epicardial muscle fibres are similarly compared those of SHR exhibit only 67 per cent of the distension displayed by

Fig. 2 Theoretical model of a transverse section of the left ventricle from SHR (upper part) and NCR (lower part). The difference between the left and the right part of the figure illustrates the changes in geometry of the respective ventricles when distended from a diastolic filling pressure of 4.5 cm H<sub>2</sub>O to 7.5 cm H<sub>2</sub>O. T represents tangential tension per unit tissue mass ( $T = (P \times r) / 2 \times w = \text{mm Hg}$ ) and  $w/l$  represents the ratio between wall thickness and lumen i.e. internal radius. The values  $\pm$  S.E. for the calculated circumferences of the inner radius ( $l_1$ ) for the "average" or mean radius ( $l_2$ ) and for the outer radius ( $l_3$ ) are given for the diastolic filling pressures of 4.5 (left part) and 7.5 cm H<sub>2</sub>O (right part). Below these figures the percentual changes in length of the respective circumferences are given as means  $\pm$  S.E.



the NCR ones (Fig. 2). Hence, even though the percentual change of the inner radius, which by common definition represents ventricular compliance, was so slightly reduced in SHR that the difference to NCR was statistically insignificant, the reduced elongation of the mean and outer wall circumferences in SHR will influence the Starling relationship as if the SHR ventricular compliance (as defined above) were reduced. In other words, most myocardial layers and particularly the epicardial ones will for a given end-diastolic pressure be less distended in the SHR left ventricle than in the NCR one.

Furthermore, the increased wall/lumen ratio of the SHR left ventricles will also reduce the tangential tension ( $T$ ) per unit wall thickness at any diastolic filling pressure ( $P$ ) since, according to Frank's modification of the Laplace law,  $T = (P \times r) / 2 \times w$ , where  $r$  is the internal radius and  $w$  is the wall thickness. The mean tangential tension per unit wall thickness thus calculated was significantly lower in the SHR ventricles for any diastolic pressure level ( $p < 0.01$ – $p < 0.001$ ) (see also Table II).

**III Frank-Starling relationship in young SHR hearts.** In the lower range of diastolic filling pressures, SHR display a reduced stroke volume compared to NCR, although no difference was obtained at DFP above 10 cm H<sub>2</sub>O (Fig. 3 left part). However, as can be seen from the right part of Fig. 3, the left ventricular stroke work of SHR is considerably greater than that of NCR at higher DFP. In this experimental setup, both resistance and pressure varies with flow, so that the afterload is increased at higher CO. An increased afterload in SHR for equal flows as in NCR may therefore indicate a greater flow acceleration in the hearts of the young SHR than in NCR.

To summarize: Adult SHR, with an 80 per cent increase in the ratio of left ventricular

## SHR-NCR 6 WEEKS OLD

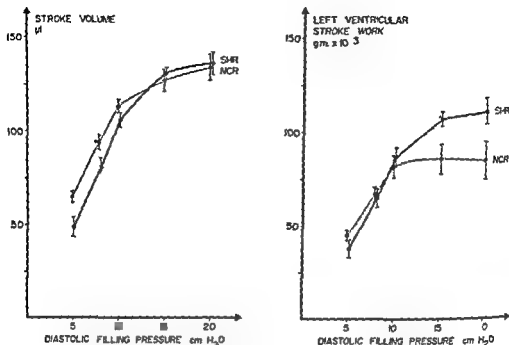


Fig. 1 Effect of increased diastolic filling pressure on stroke volume (left part) and stroke work (right part) of SHR aged 6 weeks *vs.* young SHR and age matched controls (NCR). Vertical bars represent  $\pm$  S.E.  $-p < 0.05$   $-p < 0.01$

dry weight/body weight display a 20–25 per cent reduction in stroke volume compared with NCR at DFP of 8 cm H<sub>2</sub>O. Young SHR with only a 40 per cent increase in left ventricular dry weight/body weight ratio show only a 10–13 per cent reduction in stroke volume compared with matched NCR. Thus particularly in SHR with established hypertension the Frank-Starling relationship of the hypertrophied left ventricles is altered in such a way that a higher end-diastolic filling pressure (or increased inotropy) is required to produce the same stroke work or stroke volume as in matched NCR. This appears to be due to the increased left ventricular wall thickness in SHR reducing the average diastolic compliance and tension per unit layer of cardiac muscle and thereby the functional impact of increasing DFP on stroke volume and stroke work. In young SHR representing the developing phase of hypertension the mentioned changes are proportionally less pronounced.

### Discussion

Many studies in man and in SHR have demonstrated a proportionality between the extent of increased blood pressure and left ventricular hypertrophy (*cf.* Pickering 1968 Farmer *et al.* 1974). In the present study it was illustrated that the left ventricular hypertrophy in SHR has important hemodynamic consequences by displacing the Frank-Starling relation

ship to the right in the lower range of diastolic filling pressure (DFP) the shift being largely proportional to the degree of hypertrophy. Thus in young SHR with some 40 per cent increase of left ventricular mass per body weight stroke volume was approximately 10 per cent lower at equal levels of DFP in the physiological range. Adult SHR with an 80 per cent left ventricular increase showed some 20 per cent reduction in stroke volume compared with NCR at a DFP of 8 cm H<sub>2</sub>O (see Table I). The DFP increase required in SHR to produce stroke work or stroke volumes equal to NCR was not a consequence of cardiac dilatation since left ventricular end-diastolic volumes did not differ appreciably between adult SHR and NCR in the physiological DFP range (see Table II). Further the maximal stroke work achieved at the highest DFP investigated was higher for the hypertrophied SHR left ventricles than for the controls particularly in young animals.

As discussed by Braunwald and Ross (1963) other factors than a depressed myocardial function may well be responsible for an altered Frank-Starling relationship for example a reduced diastolic compliance. The present technique for measuring left ventricular compliance (i.e. percentual ventricular volume increase for a given DFP increase) excluded the dynamical impact of the beating heart. Defined as above the diastolic compliance of the arrested SHR left ventricle was so slightly reduced compared with NCR ones that the difference was statistically insignificant. However the Starling law of the heart implies that the force of contraction is dependent on the degree of stretch on the average myocardial fibre (Starling 1918). As suggested by Meerson (1969) equal end-diastolic pressures do not ensure equality of initial fibre length when hypertrophied and non hypertrophied hearts are compared. Therefore due to the thicker wall of the SHR left ventricle the end diastolic tension per unit wall thickness will be reduced and the average muscle fibre stretching smaller even when the SHR end-diastolic volume is not significantly smaller than in NCR. This is illustrated in the theoretical model of Fig. 2 which demonstrates that the percentual elongation of the mean circumference (1 in Fig. 2) representing the average muscle fibre in the left ventricular wall becomes significantly smaller in SHR than in NCR at equal DFP increases. Thus the more the left ventricular wall/lumen ratio is increased by hypertrophy the less pronounced the myocardial prestretching will be for a given DFP increase particularly so in the outer wall layers.

When comparing the Frank-Starling curves of young and adult rats (Figs. 1 and 3) it can be seen that the myocardium of the young rats reached maximal performance at a lower DFP than that of the adult ones. Further the increased stroke work at the higher range of DFP (Fig. 3 right part) was in the young SHR not due mainly to an increased stroke volume as in adult SHR but was rather a consequence of an increased afterload. Since there was no appreciable difference between young SHR and NCR with respect to cardiac output the higher afterload in SHR suggests a greater flow acceleration when the left ventricles contract i.e. an increased contractility presumably reflecting the greater bulk of myocardium. Similar pronounced differences in left ventricular work performance were however not seen in adult SHR and NCR perhaps because the maximal DFP was limited to 20 cm H<sub>2</sub>O at which pressure level the SHR curve was still ascending. The left ventricular hypertrophy in adult SHR may however also be admixed with degenerative

myocardial changes *e.g.* by a higher content of connective tissue (Buccano *et al* 1969 Ooshima *et al* 1972)

Since the tension development rather than the performed work seems to determine cardiac energy consumption (*cf* Meerson 1969) the lower coronary flow in SHR may reflect the smaller tension developed by the SHR left ventricle than the NCR ones at equal DFP. Further, the coronary flow of both SHR and NCR increased with increasing cardiac work performance and at higher filling pressures the stroke work of the SHR ventricles exceeded that of the NCR ones despite a lower coronary flow in SHR. This indicates that the relatively lower coronary flow in SHR does not determine the reduced stroke work of the SHR left ventricles in the lower range of filling pressures. The increased coronary flow resistance of the SHR may also partly reflect a "structural autoregulation" of the coronary resistance vessels a process which seems to involve all systemic precapillary vessels in hypertension (Folkow *et al* 1973 1974).

To summarize the above results and considerations the ventricular function curves of the progressively more hypertrophied SHR left ventricles becomes displaced more and more to the right as a result of the increased wall/lumen ratio and reduced ventricular compliance so that they require higher filling pressures in the normal end-diastolic filling pressure range of 5–10 cm H<sub>2</sub>O and/or an increased inotropic influence to deliver the same stroke volume as in normotensive controls. However the maximal contractile strength of the SHR hypertrophied ventricle is increased as long as no degenerative changes are superimposed.

Much interest has been focussed on the role of the sympathetic nervous system in both labile and essential hypertension in man (*cf* Julius and Esler 1975) and in the pathogenesis of spontaneous hypertension in rats (*cf* Okamoto 1972 Folkow 1975). Labile hypertension is characterized by an increased CO but with a seemingly normal TPR (Julius and Schork 1971) whereas in established hypertension CO is normal but TPR increased (Pickering 1968). If the mechanism behind the increased blood pressure in both labile and essential hypertension were only due to a generalized increase of sympathetic tone such differences in hemodynamics would be hard to explain. Yet both in labile and essential hypertension heart rate is increased somewhat indicative of an altered neurogenic influence (Frohlich *et al* 1970 Ellis and Julius 1973). According to the present results the impact of an increased sympathetic drive to the heart in hypertension would for a given end diastolic pressure tend to increase or normalize stroke volume depending on the degree of left ventricular hypertrophy which in itself tends to displace the Frank-Starling curve to the right. Such a relationship between a decreasing stroke index and increasing left ventricular hypertrophy has been demonstrated also in hypertensive patients without signs of cardiac decompensation (Frohlich *et al* 1971). This is the case also in adult SHR where it is possible to follow more exactly the hemodynamic events during progression of hypertension.

It has however been suggested that enhanced adrenergic mechanisms play such an important role in the pathogenesis of SHR hypertension that only neurogenic elements should be involved since the elimination of all sympathetic influences in adult SHR reduces arterial pressure to almost the same levels as in equally treated NCR (Shibayama *et al* 1971). It was however recently shown that in this situation of largely equal low pressures

in SHR and NCR, TPR is still some 35 per cent higher in SHR while CO is correspondingly lower (Albrecht *et al* 1975). In accordance with the present results the structural adaptation of the left SHR ventricle would result in a lower CO in SHR than in NCR at complete cardiovascular denervation—a process which would tend to equalize central venous and cardiac filling pressures in SHR and NCR.

Human labile hypertension and early SHR hypertension are characterized by a hemodynamic pattern quite similar to that during arousal in normotensive organisms when more or less intense defence reactions are elicited. This response pattern which involves neurogenic cardiac acceleration and sympathetic vasoconstriction in all vascular beds except that of the skeletal muscles seems for genetical reasons to be more readily and more powerfully elicited in SHR thereby playing a crucial role for the initiation and progression of SHR hypertension (Hallböök and Folkow 1974, Hallböök 1975). The enhanced sympathetic influence on the SHR cardiovascular system is therefore likely to be in principal intermittent and differentiated in nature rather than tonic and generalized. Even if intermittent however an increased sympathetic discharge will raise mean arterial pressure as averaged over days and weeks and such a functional increase of the average pressure load will gradually initiate adaptive structural changes in the left heart, systemic arteries and precapillary resistance vessels largely in proportion to the pressure increase. The precapillary structural adaptation observed both in SHR and in hypertensive man implies an increased wall/lumen ratio and has the important hemodynamic consequence that increased resistance can be maintained without necessitating any increased excitatory influence (for details see Folkow *et al* 1973, 1974).

It is therefore suggested that the hyperkinetic circulatory state of labile hypertension is gradually transferred into that of a normal CO and increased TPR due to the development of the mentioned types of structural cardiovascular adaptation. That of the precapillary resistance vessels progressively contributes to the increased TPR while that of the left ventricle gradually shifts the Frank-Starling relationship to the right thereby reducing or dependent on superimposed neurogenic influences normalizing CO.

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## Effect of Temperature on Optical Density of Rabbit Blood in Dye Dilution Studies

By

NAOMI M ANDERSON<sup>1</sup> ROBERT K CREASY MICHAEL DE SWIET and  
KARI V KAHANPAA<sup>1</sup>

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### Abstract

ANDERSON N M R K CREASY M DE SWIET and K V KAHANPAA *Effect of temperature on optical density of rabbit blood in dye dilution studies Acta physiol scand 1975 94 271-277*

The optical density of rabbit blood increased on cooling. This increase was additive to the deflection produced by indocyanine green at all concentrations measured. If calibration curves were not corrected for any increase in optical density produced by cooling the blood an error (minimum 11%) was introduced in the estimation of cardiac output. The effect of temperature was investigated with three different densitometers. Its wavelength dependence did not follow the absorption spectrum of oxyhemoglobin.

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Dye-dilution curves for the estimation of cardiac output in infants and small animals are frequently quantified by performing an on-line calibration. With this calibration technique samples of arterial blood are withdrawn and allowed to stand while different amounts of dye are added to each. The samples including a blank sample to which no dye has been added are then reintroduced into a withdrawal line and passed through the cuvette of a densitometer to obtain deflections from a baseline. The baseline is established on arterial blood withdrawn directly from the subject.

In a series of experiments to determine cardiac output in pregnant rabbits by dye-dilution with indocyanine green (Cardio-Green, Hynson Westcott and Dunning, Inc., Baltimore, Maryland) we noted that our linear calibration curves in which deflection was plotted on the ordinate showed a y intercept. This finding suggested that optical density of blood increased if a delay occurred between withdrawal of the blood and its passage through the cuvette. Such a baseline shift if not corrected would introduce an error into the calculation of cardiac output. We therefore investigated the cause of the effect in rabbits

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<sup>1</sup>Present address: Department of Obstetrics and Gynecology, University Central Hospital, SF-00 900 Helsinki, Finland.



with three different densitometers. We found that blood cooled considerably while standing and that cold blood flowing through the densitometers had a greater optical density than warm blood even if no dye had been added.

### Materials and Methods

Rabbits weighing 3.4 to 5.0 kg were anesthetized *s.v.* with sodium pentobarbital (30 mg/kg) and were given supplementary xylocaine locally. Polyvinyl catheters (internal diameter 0.8 mm, external diameter 1.2 mm) were inserted into the femoral arteries at the groin and heparin (250 I.U. per kg) was administered. Blood was withdrawn through the densitometer from the femoral artery with a Harvard pump at 3.5 ml/min unless otherwise stated.

Three densitometers were used: the Waters XP 30\* (Waters Company, Rochester, Minnesota), a dichromatic instrument, and 2 monochromatic instruments: the Gilson DTL (Gilson Medical Electronics, Middleton, Wisconsin) and the Gilford 103 IR (Gilford Instrument Laboratories, Oberlin, Ohio).

The Waters densitometer was calibrated for use as follows. We withdrew a 4 ml sample of blood from the rabbit's femoral artery and either added a measured quantity of indocyanine green or did not add any dye. Arterial blood was then withdrawn through the cuvette of the densitometer and a baseline was established on a Beckman direct writing recorder. The 4 ml blood sample with or without dye was mixed, introduced into the withdrawal line and allowed to pass through the cuvette of the densitometer. After a response had been established the blood sample was removed from the system and blood was again withdrawn directly from the rabbit to check the baseline. The total time from beginning to obtain the calibration sample until its insertion into the withdrawal line to pass through the cuvette was always less than 2 min. We calibrated the densitometer at 0, 2.5, 5.0, 7.5 and 10.0 mg of dye per liter of blood.

In 4 additional studies we performed the calibration eight times at each dye concentration with blood from each of 4 rabbits. In 4 of the 8 calibrations the sample was passed through the cuvette within 2 min of its withdrawal (fast) and in the remaining 4 curves the sample was allowed to stand at room temperature for at least 15 min before being passed through the cuvette (slow).

To determine the effect of the temperature of the blood, we inserted 2 identical delay loops (polyvinyl, internal diameter 0.8 mm, external diameter 1.2 mm) between the femoral artery and the densitometer. One loop passed through air at room temperature and the other (cold loop) passed through an ice water bath. The instrument baseline was established with blood drawn through the loop at room temperature. Blood flow was then switched to the cold loop and any change from the baseline was noted. The same system was used to study the effect in rabbit plasma. For these experiments the plasma was withdrawn from a beaker in a water bath at 41°C. In order to study the effect of temperature at different flow rates the withdrawal speed of the Harvard pump was varied from 3 ml/min to 15 ml/min. The length immersed of the cold loop was altered so that the degree of cooling was the same at all flow rates.

The temperature of flowing blood was measured with a thermistor located in the catheter at the junction of the catheter and the cuvette. The thermistor was connected to Wheatstone bridge (Yellow Springs Instrument Company Inc., Yellow Springs, Ohio); the output of the bridge was displayed on the Beckman recorder. The thermistor was calibrated with flowing saline at different temperatures.

### Results

Dye calibrations with the Waters densitometer were performed on blood from 19 rabbits (15 pregnant, 4 nonpregnant). The results confirmed our previous observation of a linear correlation between dye concentration and deflection. The mean value of the correlation coefficient was 0.99. For materials that obey Beer's Law, optical density is proportional to pigment concentration. Therefore, over the range of dye concentrations used, deflection was proportional to optical density. The calibration curves always showed a positive y intercept which could be related to an equivalent dye concentration. The 19 intercepts were equivalent to a mean of 1.1 mg/l of dye (S.D. 0.39). We observed no difference in the

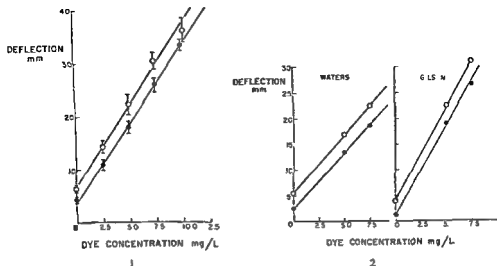


Fig 1. Difference in the slow (○) and "fast" (●) calibration curves for no dye and four concentrations of indocyanine green dye obtained with the Waters densitometer on blood from one rabbit (See text). Each point represents the mean  $\pm$  1 S.D. of 4 measurements. Regression lines were derived by the method of least squares.

Fig 2. Differences in indocyanine green dye calibrations with the Waters and Gilson densitometers with rabbit blood at 0°C (○) and at 2°C (●). Regression lines were derived by the method of least squares.

y intercepts of the curves obtained in the pregnant and nonpregnant animals. The magnitude of the y intercept was a function of the time delay between withdrawing the blood samples and passing them through the cuvette of the densitometer. In each of the additional four studies in which fast and slow calibration curves were obtained the y intercept of the two (fast and slow) calibration curves differed significantly ( $P < 0.025$  by Student's t test) (See Fig 1). The difference in the means of the intercepts was equivalent to a dye concentration of 0.55 mg/l. The slopes of the fast and slow calibration curves did not differ significantly ( $P > 0.5$ ) indicating that the increment in the optical density of the blood with the time delay was the same at all dye concentrations.

#### *Effect of temperature on the optical density of flowing blood*

Since the blood samples used for the slow calibration had been withdrawn for a longer time they were cooler than those used for the fast calibration. Therefore using the Waters densitometer we investigated the effect of the temperature of blood on its optical density. Arterial blood (8 ml) was withdrawn and divided into 2 portions: one sample was allowed to reach room temperature (22°C) and the other was immersed in an ice bath. Baselines were established as usual. The samples were then introduced into the withdrawal line. Blood that had reached room temperature showed a deflection representing an increase in optical density equivalent to 1.5 mg/l of dye. The sample that had been cooled to 0°C showed a total increase in optical density equivalent to 4.4 mg/l of dye. Part of the sample that had been immersed in ice water was then warmed to 41°C (body temperature of the

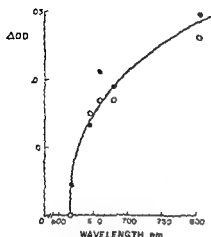


Fig. 3. Spectrum of the increase in optical density ( $\Delta OD$ ) that occurred when blood flowed through the cold loop (see text). Symbols represent results obtained on blood from 2 different rabbits.

animal). No difference was found between the optical density of the blood drawn directly from the animal and the optical density of the warmed blood.

#### *Withdrawal speed*

The effect of cooling blood to 22°C was measured with the Gilford densitometer at 805 nm at withdrawal speeds between 5 and 15 ml/min. There was no difference in the increase in optical density caused by cooling at different withdrawal speeds.

#### *Effect of temperature on calibration curves*

Dye calibration curves were obtained with the Waters and Gilson densitometers on blood that had been allowed to reach room temperature (22°C) and on blood standing in ice water. Known amounts of dye were added to the samples immediately before they were passed through the densitometer. The calibration curves at both blood temperatures for each densitometer showed a shift from the origin to higher optical density (Fig. 2). The shift was greater at the lower temperature. The curves were shifted in parallel, showing that the optical densities due to the cooling effect and to green dye are additive. Also the change in baseline due to cooling of the blood did not affect the response of the densitometer to indocyanine green. The changes in optical density with cold was in the same direction as the change from fast to slow calibration curves, confirming the supposition that the effect of time was an effect of temperature.

#### *Wavelength dependence*

We studied the wavelength dependence of the optical density change occurring when blood was cooled using the Gilford densitometer and the cold loop described previously. The wavelength of the measuring light in the Gilford densitometer was varied by replacing the 805 nm interference filter with 680, 660, 645, and 617 nm interference filters (half intensity band widths of 16–22 nm). Nonhemolyzed blood cannot be studied at wavelengths less than 617 nm with the Gilford densitometer because the optical density is extremely high.

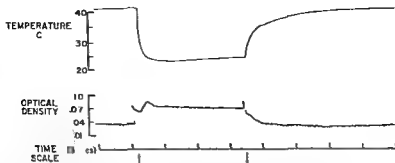


Fig. 4 Simultaneous recordings of the changes in temperature and optical density (O.D.) of blood flowing through the cold loop (see text). Recordings were made with a thermistor and the Waters densitometer. Arrows indicate introduction and withdrawal of cold loop from the system.

at the shorter wavelengths. The deflection produced by the cold blood was obtained at each wavelength. To obtain optical density changes in absolute units at each wavelength we calibrated the densitometer by inserting a neutral density filter with a nominal optical density of 0.1 in the light path while blood from the artery was flowing through cuvette. The true optical density of the neutral density filter at each wavelength had been established with a Beckman DU spectrophotometer. The densitometer deflection obtained with the neutral density filter at each wavelength was used to convert the cold loop deflection to proportionate optical density units. Results obtained on nonhemolyzed blood are shown in Fig. 3. In nonhemolyzed blood an increase in optical density was observed at wavelengths greater than 617 nm only.

The degree of cooling produced by the cold loop was measured with the thermistor. As the temperature of the blood decreased from 41°C to 22°C its optical density increased by 0.03 at 805 nm (Fig. 4).

A green dye calibration was also performed at 805 nm with the Gilford densitometer. Since the Gilford apparatus was calibrated to yield results in optical density units and since all 3 densitometers had the same sample chamber depth (0.075 cm) the optical density value at a dye concentration of 5 mg/l was used to normalize the calibration curves obtained with

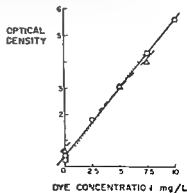


Fig. 5 Comparison of calibration curves obtained with 3 densitometers ( $\Delta$ ) Waters ( $\circ$ ) Gilford ( $\square$ ) Gilson. The three curves were normalized to the optical density 5 mg/l of dye shown by the Gilford densitometer.

the other 2 densitometers. The 3 curves are plotted in Fig. 5. The  $y$  intercept representing the increasing optical density as the blood samples were allowed to reach room temperature was comparable in all 3 densitometers.

### Discussion

Flowing blood appears to change its optical density at different temperatures. Changes in optical density were observed with all 3 densitometers to approximately the same extent and could be eliminated by warming cold blood to body temperature.

We considered that changes in the temperature of the sample might introduce changes in the properties of the densitometers. For example, the dimensions of the sample chamber might change with changes in temperature. The sample chambers of the three densitometers, however, are constructed differently. This argues against an increase in path length as the cause of the similar increase in optical density recorded by each densitometer. Also, an increase in path length suggests that the wavelength dependence should correspond to the absorption spectrum of oxyhemoglobin (Lemberg and Legge 1949) which did not occur (Fig. 3). Furthermore, there was no difference in optical density of plasma measured between 617 and 805 nm on cooling.

We next considered the possibility that changes in the temperature of the sample might alter the response of the detector. However, in the Gifford densitometer, the sample chamber is separated from the photomultiplier tube by 4.5 cm and an optical wedge; the temperature of the sample at this distance is unlikely to have any effect on the detector.

Blood flowing through narrow tubes shows axial accumulation and changes in red cell orientation (Wever 1954; Taylor 1955; Bayliss 1965; Whitmore 1967). Changes in these 2 factors can be expected with the increase in viscosity that occurs when blood is cooled (Coulter and Pappenheimer 1949). Studies on the light scattering properties of undiluted blood (Anderson and Seklj 1967) have shown that differences in optical density due to light scattering changes should increase as wavelength decreases. As shown in Fig. 3, the change in optical density did not increase with decreasing wavelength, suggesting that changes in red cell orientation did not contribute significantly to the observed changes in optical density. It is possible that optical density changes of the kind described by Wever (1954) related to axial accumulation may have contributed to the alterations we observed. These changes deserve further investigation.

Calibration of a densitometer with cold blood samples is not reliable for dye dilution studies unless allowances are made for the effect of temperature. All blood samples used for calibration, including blood for the baseline determination, should be at the same temperature. The recording machine is often adjusted so that the optical density of warm blood withdrawn from the animal before the calibration sample is added is taken as zero. In these circumstances, a deflection will be noted even when the blank sample is run due to the difference in optical density between cold and warm blood. This is the source of the  $y$  intercept. Since the effects of temperature and dye are additive at all dye concentrations, the deflection obtained with the blank sample must be subtracted from the deflection obtained at each dye concentration being used in the dye calibration. The mean value of the shift

of the baseline due to cooling of blood in our 10 calibration studies was equivalent to 1.1 mg/l of dye. If this value had not been subtracted from the calibration curve the error at a dye concentration of 10 mg/l would have been 11%. The error would increase with decreasing dye concentration.

The error in the calibration curve will falsely decrease the area under the dye dilution curve. Therefore in our experiments in rabbits this would have resulted in an overestimation of cardiac output of at least 11%.

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## Membrane Potential and Conductance during Pinocytosis Induced in *Amoeba proteus* with Alkali Metal Ions

By

JAN-OWE JOSEFSSON NELS-GUNNAR HOLMER and S ELISABETH HANSSON

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### Abstract

JOSEFSSON J O N G HOLMER and S E HANSSON *Membrane potential and conductance during pinocytosis induced in Amoeba proteus with alkali metal ions* Acta physiol scand 1975 94 278-288

An investigation of the relationship between the polarized state of the membrane and the onset and the intensity of pinocytosis was made in *Amoeba proteus*. Membrane potential and input resistance was in all instances found to decrease in approximate proportion to the number of channels when pinocytosis was induced by a variety of alkali metal ions at varying pH. Channels began to appear when the membrane was depolarized to -30 mV by the inducer of pinocytosis. With all inducers the maximum pinocytosis was encountered at membrane potentials close to zero. No positive potentials were recorded when the chloride salts of the inducing cations were used. At high concentrations of alkali ions a transient increase of the chloride permeability caused short lasting hyperpolarizations of the membrane. Inhibition of pinocytosis by  $\text{Ca}^{++}$  was accompanied by an increase of input resistance and membrane potential. The selectivity of the membrane to different alkali metal ions observed as changes in pinocytosis intensity, membrane potential and input resistance was found to vary with the concentration of the inducer and with the  $\text{Ca}^{++}$  concentration of the extracellular solution. Displacement of membrane bound  $\text{Ca}^{++}$  appeared to decrease the field strength of charged groups in the membrane altering its selectivity among alkali cations. The formation of pinocytotic channels is suggested to require translocation of  $\text{Ca}^{++}$  from the membrane into the cell and would therefore be closely related to the electrical properties of the amoeba.

The effects of external  $\text{Na}^{+}$  and  $\text{K}^{+}$  on the transmembrane potential of *Amoeba proteus* has been studied by Bingley and Thompson (1962) and by Josefsson (1966). Increasing the external concentration of either of these ions independently lowered the potential and induced pinocytosis in the cells. In both respects  $\text{Na}^{+}$  was less potent than  $\text{K}^{+}$ . The decrease in membrane potential was accompanied by a reduction in input resistance of the cell.

In a recent study (Josefsson 1968) it was observed that monovalent cations induced pinocytosis, the order of effectiveness being  $\text{Cs} > \text{K} > \text{Na} > \text{Li}$ . Divalent cations were less active inducers and  $\text{Ca}^{++}$  and  $\text{Mn}^{++}$  in that order were shown to reduce the sensitivity of the cell to the inducer.  $\text{Ca}^{++}$  and  $\text{Mn}^{++}$  were themselves without pinocytosis inducing effects.

An increase in the sensitivity of the amoeba towards all inducing media was noted when calcium chelating substances were added to the medium or used for the pretreatment of the cells. Inhibition of pinocytosis was also observed when the hydrogen ion concentration in the medium was decreased although this effect was limited to inducers of low affinity e.g. to  $\text{Na}^+$ ,  $\text{Li}^+$  and to some of the bivalent cations.

Recently Brandt and Freeman (1967) observed that  $\text{Ca}^{++}$  besides its inhibitory effect on pinocytosis increased the effective membrane resistance of *Amoeba proteus*. Thus there were reasons for regarding  $\text{Ca}^{++}$  and  $\text{H}^+$  to be of significance for the membrane conductance as well as for the hypothetical pinocytosis receptor. These findings prompted us to study the effects of  $\text{Ca}^{++}$  and pH on membrane potential and input resistance during pinocytosis induced by different inorganic cations.

## Methods

*Amoeba proteus* fed on *Tetrahymena pyriformis* and starved 2 days before an experiment were cultured in Pringsheim solution at 22°C according to the technique of Prescott as modified by Chapman and Andresen (1958). The composition of the culture solution and the technique for intracellular recording of membrane potentials were given in an earlier report (Josefsson 1966).

Electrodes, filled with 3M KCl were selected for low tip potentials. In some experiments in media of low electrolyte concentration tip potentials were reduced by the addition of 0.05 mM  $\text{Th}(\text{NO}_3)_4$  to the 3M KCl-solution in which the electrodes also were stored.

Input resistance was initially measured using the square pulse technique (Josefsson 1966). Later however a voltage clamp technique was used which made it possible to measure input resistance at a preset membrane potential. The latter method had technical advantages which made it suitable for the present study. A diagram of the voltage clamp circuit is given in Fig. 1. A single side emitter follower of FET type with high input impedance was used for measuring the membrane potential. The bath was held near ground potential (virtual ground) through a bath electrode connected to the inverting input of the current measuring amplifier. The maximum current to be measured with this device was  $15 \cdot 10^{-4}$  A. With the electrode used it was possible to successfully apply command signals (voltage in Fig. 1) of  $\pm 100$  mV except when the input resistance of the cell was below 0.2 M $\Omega$ . The command signals were taken stepwise from a constant voltage source. The operational amplifiers (Philbrick Type USA) required  $\pm 15$  V power supply.

The input resistance of the amoeba was calculated from the positive or negative current necessary to be injected when the membrane potential was increased by steps of 10 mV from 0 to 100 mV. At low membrane resistances a smaller potential swing was used in order to avoid leakage of the cell membrane. Although the current-voltage relationship did not obey Ohm's law it was considered justified to treat the resistance as ohmic for the purpose of this study.

During an experiment the cells were put in a chamber filled with Pringsheim solution. The electrodes were inserted and membrane resistance was measured by clamping stepwise from 0 to 100 mV within 2 s. Thorough change of Pringsheim for test solution was accomplished within 30 s by emptying and filling the bath four times. Approximately 40 ml of the test solution was used for 1 experiment. After allowing the cell to reach a steady membrane potential the transmembrane potential was held at this value by the voltage clamp. Unless stated otherwise all measurements of resistances were made within 5 min after the test solution was applied to the cell. The input resistance of the cells in Pringsheim solution was  $9.6 \pm 0.75$  M $\Omega$  (mean  $\pm$  S.E.) in 53 cells with the voltage clamp technique and  $10.5 \pm 0.70$  M $\Omega$  ( $n=54$ ) when measured with square pulses. The difference is not significant.

The technique for estimating pinocytosis intensity was the one described by Josefsson (1968). Numerical values for pinocytosis intensity are given as the number of channels developed per amoeba per minute during the first twenty minutes of the pinocytosis cycle. All electrical and morphological studies were made at room temperature (25°C). pH was adjusted with HCl or LiOH. The solutions used were made from chemicals of analytical grade. Thallium chloride was a Merck Suprapur product. Deionized glass distilled water was used throughout the experiment.





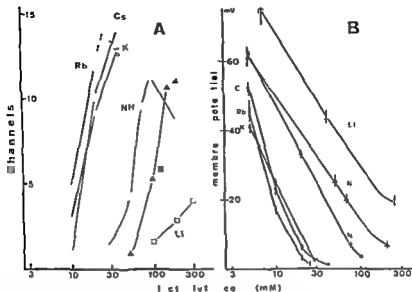


Fig. 2A Pinocytosis versus concentration for univalent cations at pH 5.7. The values for Rb, Cs and K and the values for NH<sub>4</sub>, Na and Li were obtained from 2 separate batches of cells.

Fig. 2B Membrane potential versus concentration of univalent cations at pH 5.7. The measurements for each point were made in at least 15 cells. Vertical bars denote  $\pm$  S.E.

was less than that induced by similar concentrations of K<sup>+</sup> and Rb<sup>+</sup> (Fig. 3). Furthermore in the beginning of the pinocytosis cycle Cs<sup>+</sup> also was less effective than K<sup>+</sup> and Rb<sup>+</sup> in inducing channels at the 10 mM concentration level (Table 1A). These differences between Cs<sup>+</sup> on one hand and K<sup>+</sup> and Rb<sup>+</sup> on the other could be explained by the effects of Ca<sup>++</sup> on membrane conductance and pinocytosis. Ca<sup>++</sup> being much more effective in blocking the membrane conductance increase produced by Cs<sup>+</sup> and Na<sup>+</sup> than that caused by the

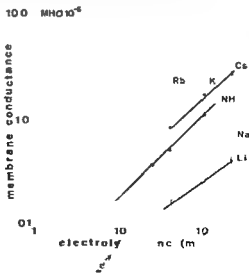


Fig. 3 Membrane conductance versus concentration of univalent cations at pH 5.7. The chlorides of Rb, K, Cs, Na and Li were at pH 5.7 while NH<sub>4</sub> was at pH 5.5. Where more than four measurements were available the  $\pm$  S.E. are given as vertical bars.

TABLE I A The number of channels observed per amoeba during the first six minutes after application of test solution. The number of experiments is given in brackets.

■  $\text{Ca}^{++}$  inhibition of the pinocytosis induced by  $\text{Ca}^{++}$  and  $\text{K}^{+}$ . The pinocytosis intensity in solutions containing 0.1 mM  $\text{CaCl}_2$  is given as percent of the intensity found in corresponding inducing solutions with no calcium added. Values are the mean of 2 experiments.

		10 mM		20 mM				
A	RbCl	14.7 (9)		25 (4)				
	KCl	9.7 (9)		27 (4)				
	CaCl	1.7 (9)		25 (4)				
B	KCl	20 mM	pH 5.0	73	CaCl	20 mM	pH 5.0	11
	KCl	0 mM	pH 5.7	62	CaCl	20 mM	pH 5.7	37*
	KCl	0 mM	pH 6.5	48	CaCl	20 mM	pH 6.5	23

presence of  $\text{K}^{+}$  and Rb (Table II). The same applied to the intensity of pinocytosis.  $\text{Ca}^{++}$  was more effective at blocking  $\text{Cs}^{+}$  induced pinocytosis than pinocytosis caused by similar concentrations of  $\text{K}^{+}$  (Table I). Inhibition of pinocytosis in the  $\text{Ca}^{++}$  containing solutions was increased by pretreatment of the cells in  $\text{Ca}^{++}$  free solution (0.3 mM NaCl pH 5.7 for 30 min). Considering the great differences between  $\text{Cs}^{+}$  and  $\text{K}^{+}$  when  $\text{Ca}^{++}$  is present it appears that pinocytosis induced by these ions could depend on the  $\text{Ca}^{++}$  content of the amoeba membrane at the moment of exchange of solutions.

#### Potassium induced pinocytosis and related membrane potentials

The pinocytosis induced by  $\text{K}^{+}$  was estimated at four different values of pH (Fig. 4A) and the effect of pH and  $\text{K}^{+}$  ion concentration on the membrane potential was measured in parallel cultures of amoeba (Fig. 4B). In the presence of  $\text{K}^{+}$  concentrations which completely depolarized the membrane of the amoeba the greatest number of channels occurred at pH 7.0, 5.8 and 4.5. The most intensive pinocytosis and greatest depolarization occurred at pH 4.5. At pH 3.0 very little pinocytosis was observed and the cell was at this pH only partially depolarized irrespective of high potassium concentration.

The membrane potentials recorded at  $\text{K}^{+}$  concentrations above 50 mM were complicated by the observation that the insertion of the microelectrode produced a transient hyperpolarization of the cell of 10–100 seconds duration. The hyperpolarizing potential starts

TABLE II Effects of membrane resistance measured with voltage-clamp technique in cells immersed in different solutions at pH 5.7. The number of experiments is given in the column marked (n).

Extracellular solution		(n)	$M\Omega \pm S.E.$
CaCl	25 mM	11	$1.8 \pm 0.78$
CaCl	75 mM	15	$5.2 \pm 0.47$
KCl	25 mM	12	$1.5 \pm 0.21$
KCl	25 mM	11	$2.2 \pm 0.76$
RbCl	25 mM	4	$1.8 \pm 0.29$
RbCl	25 mM	7	$2.1 \pm 0.16$
NaCl	40 mM	4	$9 \pm 0.74$
NaCl	40 mM + 2.5 mM $\text{CaCl}_2$	4	$9.3 \pm 0.74$

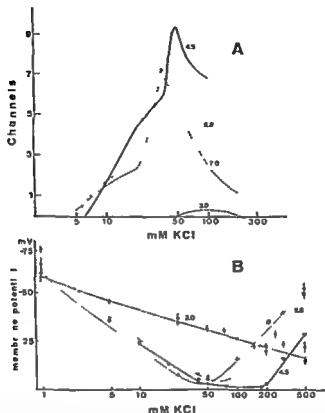


Fig. 4A Intensity of pinocytosis induced by KCl at different pH values indicated in the figures. The curves are fitted through the means at the following  $K^+$  concentrations 5 10 30 50 100 and 300 mM (3 to 8 measurements/concentration). The measurements at pH 3.0 are from a single experiment. Fig. 4B Membrane potential in amoeba immersed in 1 to 300 mM KCl at different pH values. The mean values for 10–15 amoebae is given with  $\pm$  S.E. Measurements from different cultures are the cause of two readings at some concentrations. The curves with positive slopes (KCl 50–300 mM) are due to hyperpolarizing potential (see Fig. 5).

immediately but developed slowly and when input resistance measurements were made an increase in membrane conductance was observed. Fig. 5A illustrates a typical potential which developed as a result of impaling the cell with a second electrode. The input resistance was low until the repolarization began. Similar potentials could be produced by intracellular stimulation using depolarizing currents (Fig. 5B). Hyperpolarizing potentials were also observed to occur spontaneously frequently in connection with increased cellular motility.

To investigate the cause of the transient hyperpolarization which accompanied the insertion of the microelectrode a study was made in the presence of 300 mM  $K^+$ . The possibility that the potential change was due to chloride current was investigated in media of 300 mM  $K^+$  by increasing the concentrations of  $Cl^-$  and ethanesulphonate in varying proportions. In such an experiment the membrane acted as a chloride electrode (Fig. 6). The slope of membrane potential against  $(Cl^-)$  was approximately 60 mV per tenfold change of  $(Cl^-)$  concentration at room temperature. Pinocytosis intensity was not influenced by these

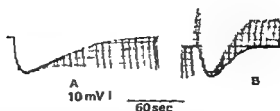


Fig 5 Continuous record of membrane potential on which is superimposed electrotonic potentials resulting from injection of square pulses (current  $10^{-7}$  A) of  $\sim 0.0$  msec duration. The amoebae were in 300 mM KCl +  $\sim 5$  mM  $\text{CaCl}_2$ . Resting potential was zero mV. Amplitude of electrotonic potential is proportional to effective input resistance. Vertical and horizontal bars indicate 10 mV and 1 min respectively. A Hyperpolarizing potential after insertion of second electrode. B Similar potential induced in this case by outward current from the intracellular stimulating electrode. The transient potentials were accompanied by a decrease of input resistance.

potentials. Hyperpolarizing potentials also appeared in  $\text{Ca}^{++}$  free K<sup>+</sup> solutions but were of higher amplitude and of longer duration in the presence of  $\text{Ca}^{++}$ . In this respect  $\text{Sr}^{++}$  could not substitute for  $\text{Ca}^{++}$  (Fig. 6). The presence of group 1 A metal cations favoured the development of the potential (Table III). The order of efficacy in this respect seemed to be that of free solution mobilities ( $\text{Cs} > \text{Rb} > \text{K} > \text{NH}_4 > \text{Na} > \text{Li}$ ). Pretreatment of the amoeba with a solution containing a calcium chelator inhibited the potential as well as the rush of cytoplasm towards the electrode which usually followed the impalement.

Addition of  $\text{Ca}^{++}$  to the external medium of *Amoeba proteus* inhibits induced pinocytosis in a competitive manner (Cooper 1968; Josefsson 1968) and increases the effective resistance of the cell (Brandt and Freeman 1967). To investigate concomitant changes in the membrane potential,  $\text{Ca}^{++}$  was added to a solution containing NaCl (100 mM at pH 7.0 or 200 mM at pH 5.8) or KCl (40 mM at pH 5.8 or 7.0). These pH's and ionic concentrations were chosen because they induced a high level of pinocytosis and were within the ascending part of the Jose response curves for respective ion. In these media the addition of  $\text{Ca}^{++}$  caused an increase in the membrane potential of the cells as shown in Fig. 7A. The time course of the

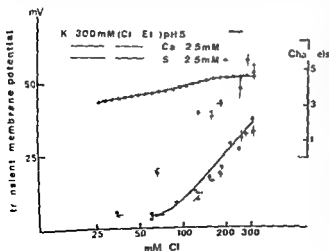


Fig. 6 Transient membrane potentials recorded in 300 mM K<sup>+</sup> salt solutions at pH 5.0. The chloride concentration (abscissa) was varied by substitution of chloride for ethanesulphonate. The addition of  $\text{Ca}^{++}$  made the potentials appear at a lower chloride concentration. Extrapolation of this curve indicated an intracellular chloride concentration of approximately 25 mM. The broken line in the middle of the graph shows the level of pinocytosis intensity in the 300 K<sup>+</sup> 5  $\text{Ca}^{++}$  solution. The slightly weaker pinocytosis at low chloride concentration is probably due to the  $\text{Ca}^{++}$  binding activity of the ethanesulphonate ions.

TABLE III Transient potentials (mV) as measured in 500 mM of the chlorides of different alkali ions at pH 5.7. The means  $\pm$  S.E. are given. The number of experiments is within the brackets

Cs	57.4 $\pm$ 2.13 (17)	NH <sub>4</sub>	27.7 $\pm$ 1.42 (18)
Rb	56.2 $\pm$ 3.58 (19)	Na	17.4 $\pm$ 0.79 (33)
K	44.4 $\pm$ 1.81 (24)	Li	16.2 $\pm$ 1.03 (33)

repolarization was similar for the Na<sup>+</sup> and K<sup>+</sup> solutions when the experiments were performed at pH 5.8. At neutral pH, however, the effect of Ca<sup>++</sup> was more pronounced in 100 mM NaCl than in 40 mM KCl. As shown by Fig. 7B, increasing concentrations of Ca<sup>++</sup> gradually inhibited pinocytosis induced in the presence of KCl or NaCl solutions. With the exception of pinocytosis induced by K<sup>+</sup> at pH 7.0, 1 mM Ca<sup>++</sup> completely inhibited pinocytosis. The inhibition seemed to follow a linear course in the semi log plot. A similar relationship was found for membrane potential versus increasing Ca<sup>++</sup> concentrations. In the experiments with the addition of Ca<sup>++</sup> at pH 5.7, inhibition of pinocytosis occurred at a level of membrane potential lower than that necessary to induce pinocytosis, 30 mV (compare Figs. 7A and 7B with 2A and 2B).

### Discussion

The data reported on the potentials measured in *Amoeba proteus* are in accordance with the picture that the amoeba membrane is permselective to cations (Josefsson 1966). The membrane potential was dependent on the concentration and species of the monovalent cation in the extracellular solution and on the pH of the solution. Thus a tenfold increase of K<sup>+</sup> concentration at pH 5.7 caused a depolarization of 50 mV. This level of depolarization, which is larger than that earlier reported (Josefsson 1966), probably reflects a different technique for impaling cells. As a comparison, the values for resting potentials in Pringsheim was around -80 mV instead of the -65 mV reported earlier.

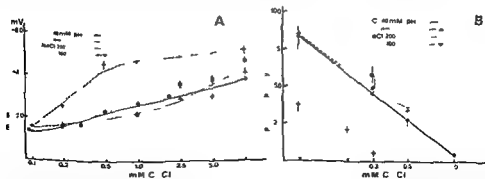


Fig. 7A. Membrane potential (ordinate) measured in KCl 40 mM (pH 5.8) and pH 7.0) NaCl 100 mM (pH 7.0) and 100 mM (pH 5.8). To these solutions, CaCl<sub>2</sub> was added to increase its concentration up to 10 mM (abscissa). Vertical bars denote  $\pm$  S.E.

Fig. 7B. Inhibition of pinocytosis by Ca<sup>++</sup>. Pinocytosis is measured as percent of channels observed in respective inducing solution with no Ca<sup>++</sup> added. Each point is a range of 4-11 experiments. The vertical bars indicating two times the standard error of the means.

The slope for depolarization against increasing concentrations of  $K^+$  (pH 5.7) approached that predicted by the Nernst formula (i.e. the membrane is a  $K^+$  electrode). At high hydrogen ion concentrations (pH 3.0) the slope was markedly decreased which may signify that the membrane is becoming permeable to anions.

During the transient hyperpolarizing response to mechanical stimulation the amoeba behaved like a chloride electrode for  $[Cl^-] = 25$  mM and the amplitude of the hyperpolarizing potential increased in the sequence determined by the ionic crystal radius of the cation present. Consequently the original exclusion of the Cl anions from passing through the membrane decreased as the cation radius increased (i.e.  $Cs > Rb > K > Na > Li$ ). Simultaneously with the change in potential a decrease in transmembrane resistance was observed. The presence of  $Ca^{2+}$  but not  $Sr^{++}$  increased the sensitivity of membrane potential to Cl. This might reflect the stabilizing effects of  $Ca^{2+}$  (not demonstrated by  $Sr^{++}$ ) which may involve a "sealing" of the membrane around the microelectrode tip after penetration and thus a decreased permeability to cations. The potentials observed by Lassen *et al.* (1974) after puncture of *Amphiuma* red cells were increased by  $Ca^{2+}$  which was suggested to have an intracellular effect. Similarly  $Ca^{2+}$  diffusing into the amoeba may increase the Cl permeability of the membrane and subsequent sequestering of  $Ca^{2+}$  may explain the transient nature of the potential.

The study of the input resistance as measured with the voltage-clamp technique gave no evidence for abrupt increase of conductance when the concentration of the external electrolyte was increased to the level where pinocytotic channels develop. There was an approximate quantitative relationship between the number of channels induced and the value of the input resistance. Channel formation commenced at an input resistance of 4 M $\Omega$  and intense pinocytosis (i.e.  $\sim 10$  channels per amoeba and minute) was observed when it was approx. 1.5 M $\Omega$ .

The order in which cation species induce pinocytosis and the membrane permeability to these cations could in the light of recent theories presented by Eisenmann (1962, 1964) give information on the nature of negative membrane sites. As shown by Eisenmann (1963) the sequence of selectivity for cation species in several biological systems appears to follow the "field strength theory" of ionic specificity of glass electrodes (cf. Diamond and Wright 1969). The permutations of sequence for Cs, Rb and K obtained in the studies of pinocytosis (Fig. 2 A), membrane potential (Fig. 2 B) and transmembrane conductance (Fig. 3) were in accordance with some of those suggested by Eisenmann. As the cation concentration was increased these three parameters exhibited a gradual transition of sequence permutation, IV (K > Rb > Cs)  $\rightarrow$  III (Pb > K > Cs)  $\rightarrow$  II (Rb > Cs > K) indicating a decreased anionic field strength of the cation-detecting structures present in the membrane. Sequence I (Cs > Rb > K) observed by Joffsson (1964) was never attained in this study. The curve for conductivity passed through the first (IV  $\rightarrow$  III) and pinocytosis passed through the second (III  $\rightarrow$  II) of these transitions while the selectivity order for membrane depolarization included both (IV  $\rightarrow$  III  $\rightarrow$  II). In the membrane potential measurements the transition in sequence permutation occurred at lower concentrations than in the conductance studies. This was probably due to the difference in duration of the two types of experiments and might indicate an effect of  $Ca^{2+}$  on the ionic selectivity of the membrane. The  $Ca^{2+}$  concentration of the

membrane is likely to decrease with time in the medium. Likewise a decrease of the membrane bound  $\text{Ca}^{++}$  can be expected when the external concentration of alkali cations is increased. A conformational change in the membrane of the amoeba during induction of pinocytosis is suggested from two recent observations. Swelling of the amoeba membrane was observed by Brandt and Freeman (1967) when the input resistance was low and the pinocytosis intensive and a general increase of permeability was found to be produced by cations in proportion to their efficacy as inducers (Brandt and Hendil 1970, 1972). The altered membrane selectivity which occurred when the concentration of cations was raised might indicate that structural changes are caused by univalent-divalent ion exchange in the membrane. Release of  $\text{Ca}^{++}$  from the membrane into the cytoplasm could be the mechanism which relates permeability and pinocytosis to the conformational changes.

In general the correlation between intensity of induced pinocytosis and membrane polarization was good. Inhibition of pinocytosis by changes of  $\text{Ca}^{++}$  concentration in the medium or by altering the pH of the external solution were in all instances accompanied by an increase of membrane potential. The channels appeared when the potentials were 30 mV or less but the addition of  $\text{Ca}^{++}$  seemed to inhibit pinocytosis before the membrane potential was brought back to this level. Moreover at a given concentration of alkali metal cations the permutation sequence number was higher for pinocytosis than for the membrane potential. This could mean that exchange of ions occurs at sites relevant for pinocytosis but different from those which determine the permeability characteristics of the membrane. A simultaneous comparison between the bioelectrical parameters and the pinocytosis intensity measured on the same cell would settle this question. It has been claimed from the study of pH optima of the cation induced pinocytosis (Josefsson 1968) that certain membrane groups may constitute receptors for certain cation inducers of pinocytosis. Thus  $\text{Tris}$  and  $\text{Na}^+$  will induce pinocytosis at pH 7 while  $\text{K}^+$  is most effective at pH 5.6. Without postulating specific receptors for simple cations this finding may be explained in terms of the field strength theory by the difference in sequence permutation among cations in membranes of high (at pH 7) and low anionic field strengths.

Depolarization of the amoeba membrane probably starts a chain of events about which we have little knowledge leading to invagination of the cell membrane.  $\text{Ca}^{++}$  is required for channel formation and regulates the membrane potential and input resistance of the cell. Displacement of  $\text{Ca}^{++}$  from the membrane causing a change in these three parameters. Translocation of  $\text{Ca}^{++}$  from the cell membrane into the cytoplasm may be an intermediate step in the chain of events between addition of the inducing cation and channel formation. This mechanism may explain why the pinocytosis induced by alkali metal cations is reflected by the electrical properties of the cell.

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## The Pressure-Flow Relationship of Different Nephron Populations in the Rat

By

Ö KALLSKOG L, Ö LINDBOM, H R ULFENDAHL and M WOLGAST

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### Abstract

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The mechanisms behind the autoregulation of the total renal blood flow and the glomerular filtration rate are unclear. In this investigation a modified microsphere technique was applied to measure the blood flow at different depths in the renal cortex during normotensive and hypotensive conditions. No autoregulation was found in the outer cortex while it was well pronounced in the inner one. During similar conditions glomerular capillary pressure, walling point pressure and intratubular pressure were recorded. By combining these results with the blood flow data the preglomerular and postglomerular resistances were calculated. It was then found that the preglomerular resistance decreased and the postglomerular resistance increased when the blood pressure was lowered. The results indicate a redistribution of blood flow from the outer parts to the inner parts of the cortex when the blood pressure is decreased. The redistribution of the blood flow might explain the well known linear relationship between the arterial pressure and the urine flow. The single nephron filtration rate of the outermost glomeruli could be calculated and the results seem to indicate a non-equilibrium at the end of the glomerular capillaries.

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The pressure-blood flow relationship in the kidney is characterized by an essentially unchanged blood flow when the perfusion pressure in the renal artery varies between some 70 and 200 mm Hg (Thurau 1964, Waugh 1964, Hinshaw 1964, Loyning 1971). This phenomenon has been referred to as the autoregulation of the renal blood flow. Also the glomerular filtration rate (GFR) is autoregulated within approximately the same pressure range (Forster and Maes 1947, Selkurt *et al* 1949, Thompson *et al* 1957). It has been concluded that the mechanism behind the autoregulative response is localized in the afferent arterioles.

The finding of an autoregulation of the total renal blood flow does not obviously rule out the possibility that some of the nephron populations do not exhibit this response.

In the present study the blood flow in different nephron populations during normo- and hypotensive conditions was investigated by means of a modified microsphere technique with microdissection of single glomeruli. The renal perfusion pressure was changed by an aortic clamp between the two renal arteries. The results indicate that the deeper the glomeruli the more effective the autoregulation of blood flow. The superficial glomerular blood flow was nearly linearly related to the perfusion pressure. This might then mean that a de

creased perfusion pressure leads to a redistribution of the blood flow from the superficial salt loosing glomeruli to the deep salt saving ones (Goodyear and Jaeger 1955 Barger 1973). This phenomenon might at least partly explain the well known linear relation between urine and sodium excretion and the perfusion pressure (Selkurt 1951).

In an additional study measurements of the glomerular capillary pressures and the walling point pressures were made. By combining these results with those of the blood flow measurements in the superficial glomeruli the pre- and postglomerular resistances were calculated. During decreasing blood pressures the afferent arteriolar resistance was found to decrease while the efferent arteriolar resistance increased.

### Material and Methods

All experiments were carried out on male Sprague Dawley rats weighing between 165 and 300 g. The rats were anaesthetized with intraperitoneal administration of Inactin® (Chemical Fabrik Promonta GmbH Hamburg, West Germany) in a dose of 120 mg/kg b.wt. tracheostomized and placed on a servo-controlled heating pad. The left kidney was exposed by a flank incision and placed in a lucite cup. The kidney was covered with a rice paper drenched with mineral oil. A clamp was placed on the aorta between the renal arteries. In the microsphere experiments a catheter was inserted into the right carotid artery and placed with its tip at the aortic root just above the aortic valves. Blood was drawn from a catheter in a femoral artery with a suction pump (Syringe pump Model 351 Sage Instrument USA) at a rate of 0.6 ml/min while the arterial pressure was monitored in the other femoral artery. In some experiments the pressure was measured directly in the renal artery via a 200  $\mu$ m steel cannula (Källskog *et al.* 1975). This pressure was only slightly below the one which was recorded in the femoral artery.

The spheres labelled with  $^{141}\text{Ce}$  and  $^{85}\text{Sr}$  (3M Co. Saint Paul Minn. USA) were separated by a sedimentation procedure in large glass cylinders as described previously (Källskog *et al.* 1975) by which the size scatter could be reduced to about half of the original value ( $15 \pm 5 \mu\text{m}$ , mean  $\pm$  S.D.). About 0.5 ml spheres of either kind (as a suspension in 0.5 ml of plasma) were injected. The injection time was about 20 s. The suction pump was started just before the injection and was stopped about 15 s after the injection of the sphere suspension.

The first microsphere injection was performed during control normotensive conditions as a rule with the use of the  $^{141}\text{Ce}$  labelled batch. The perfusion pressure was then reduced to the desired level followed by the injection of the  $^{85}\text{Sr}$  labelled spheres. After completion of the experiment the abdominal aorta was catheterized with a relatively large catheter through which liquid silicon rubber (Microfil, Canton Biomed Prod. Inc. Box 2017 Boulder Colorado 80302 USA) was injected in order to visualize the renal vascular tree.

The kidneys were excised and macerated at 40°C for 30 min in 50% hydrochloric acid. After rinsing the kidneys were embedded in carboxymethyl cellulose, frozen to  $-20^\circ\text{C}$  and in a microtome cut into 400  $\mu\text{m}$  thick sections. From these sections 50 or more superficial and true juxtamedullary glomeruli respectively were sampled (Bankir 1973) and analyzed with respect to their  $^{141}\text{Ce}$  and  $^{85}\text{Sr}$  activities in a gamma spectrometer (Nucab AB P.O. 2309 S-421 02 Västra Frölunda Sweden). The true juxtamedullary glomeruli were defined as those giving rise to vasa recta. The sections were then cut perpendicularly to the striatal direction in four cortical zones as according to Fig. 1. The glomeruli in the deepest part are both true juxtamedullary ones but also includes those which do not give rise to vasa recta.

The blood flow in the samples was calculated by multiplying the sampling rate of 0.6 ml/min with the sample activity over the activity in the constantly withdrawn reference blood sample. For cardiac output determinations the sample activity was replaced by the total amount of the activity injected. The blood flow value then refers to cardiac output minus the coronary blood flow. The total blood flow in the region for which a zone is representative could then be calculated by multiplying the total renal blood flow with the quotient between the activity of this zone over the summated activity of the four zones.

#### Micropuncture procedure

In further experiments the pressure characteristics in the cortical vascular tree and in the proximal tubules were investigated. The pressures were determined with a servo-controlled counterpressure measuring

## GLOMERULI

I

II

IIIa

IIIb



## ZONES

1

2

3

4

Fig. 1 The picture shows a microphotograph of the cortical part of the rat kidney. The vascular bed was filled with silicone rubber and the 300  $\mu$ m thick section was previously immersed into solutions with an increasing concentration of ethanol and then in methylene salicylate. The different types of glomeruli I, II, IIIa and IIIb are noted to the left. The brackets to the right show the different zones cut from the sections.

device (according to Wiederhielm (1964) as modified by Intaglietta (1970)) connected to a sharpened glass capillary with a tip diameter of about 4  $\mu$ m. Glomerular capillary pressure was measured indirectly by the stop-flow method according to Gertz (1966). For this purpose an early proximal tubule was punctured with a relatively thick cannula through which mineral oil was injected until the glomerular filtration ceased. The stop flow pressure thus induced was measured with a second pressure cannula inserted proximally to the oil column. From this pressure the glomerular capillary pressure was obtained by adding an oncotic pressure of 22 mm Hg corresponding to a systemic plasma concentration of 6 per cent (Landis and Pappenheimer 1963). The lowest possible perfusion pressure investigated was about 50 mm Hg under which pressure the filtration vanished and the tubules were collapsed.

## Results

*Blood flow data*

All blood flow data are brought together in Table I and are summarized in Fig. 2, 3 and 4.

Fig. 2 shows the relation between the perfusion pressure and the total renal and regional blood flow in the left experimental kidney. Experiments with weak or no autoregulation with respect to the total renal blood flow are excluded (marked with an asterisk in the table).

A further treatment of these excluded experiments showed that this weak autoregulation was valid even for the regional blood flow and in addition was mainly not accompanied by a redistribution of the blood flow. The lines drawn represent the best fit to the parabolas.

TABLE 1 Perfusion pressure, cardiac output, total and regional renal blood flows during control, normotensive conditions and during the reduction of the perfusion pressure to the left experimental kidney. Experiments denoted with an asterisk represent non autoregulating kidneys with respect to the total renal blood flow.

Rat weight g	Perfusion pressure mm Hg	Cardiac output ml/min 100 g BW		RBF exp kidney ml/min 100 g BW		Blood flow in cortical zones experimental kidney $\mu$ /zone				Superficial glomerular flow ml/min 100 g BW		Juxtamedullary glomerular flow ml/min 100 g BW		RBP control kidney ml/min 100 g BW				
		pre clamp	clamp	pre clamp	clamp	1	2	3	4	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	
318	118	78	24.3	21.9	1.83	1.38	206.2	83.3	104.5	68.1	106.5	100.4	76.9	75.9	1.89	1.60		
309	100	74	27.1	25.4	2.20	1.91	151.2	95.1	100.5	92.5	73.8	94.7	72.0	90.6	1.71	1.39		
311	117	54	32.6	21.4	1.60	0.90	136.2	54.6	66.7	43.9	40.6	43.5	39.0	44.1	2.71	1.98		
211	102	44	34.6	27.7	2.36	0.96	197.6	66.5	139.7	51.9	91.9	55.6	81.1	51.4	2.40	1.72		
290	117	31	29.0	18.7	2.86	0.72	317.4	28.0	239.3	33.2	174.2	36.8	104.6	25.7	1.66	1.62		
290	107	56	34.9	35.7	2.38	1.72	243.2	123.0	115.6	99.0	88.5	99.7	59.1	80.8	2.90	2.03		
*83	120	17	46.3	12.5	3.02	0.14	393.9	22.4	192.9	10.4	139.8	10.1	113.0	10.1	1.86	0.95		
270	124	78	37.8	28.2	2.03	1.71	184.1	106.0	106.7	98.3	80.2	94.4	53.7	63.5	2.29	1.54		
263	102	19	31.3	17.4	3.52	0.24	215.1	6.3	145.5	10.7	77.0	8.3	94.9	8.0				
263	102	19	31.3	17.4	2.11	0.20	222.5	31.2	162.4	5.6	93.7	7.3	69.5	5.1				
265*	113	81	33.0	32.5	2.65	1.99	190.8	80.0	106.8	89.4	86.5	77.2	83.1	81.0	74.3	2.37	1.90	
307	105	21	20.6	22.7	2.40	0.22	94.8	11.2	90.0	16.7	75.2	7.1	50.6	14.2	1.92	1.39		
285	110	21	42.8	19.5	3.13	0.28	211.7	14.6	121.6	14.3	98.2	12.0	93.0	15.3				
285	110	21	42.8	19.5	2.79	0.17	275.6	5.0	102.1	3.9	64.9	5.8	50.2	3.2				
298	125	82	42.2	30.2	3.63	2.86	311.9	134.1	107.9	103.1	59.1	87.8	60.5	65.4	189.3	1.51	1.34	
302	125	80	30.6	19.2	1.68	1.12	82.0	33.9	48.8	37.7	32.2	31.8	23.2	20.4	110.9	1.63	0.85	
313	127	76	23.9	0.9	1.88	1.28	46.3	33.0	52.2	39.7	41.2	43.4	33.8	31.1	93.9	1.79	1.44	
306*	105	75	27.3	27.0	2.53	1.83	94.9	56.7	87.7	60.0	46.6	49.2	68.5	71.2	178.8	1.43	1.65	
*86	130	80	33.5	27.8	1.90	1.51	78.3	52.3	52.4	58.5	34.0	38.3	26.9	24.6	115.7	0.75	0.04	
269*	116	91	33.5	30.5	2.15	1.65	160.5	57.8	110.0	105.7	54.0	76.4	32.9	41.4	137.9	0.75	0.04	
*89	132	57	30.2	23.0	1.69	1.8	43.7	22.2	36.3	27.4	23.0	23.7	32.5	30.2	130.5	0.75	0.04	
271	127	50	29.9	21.1	1.54	0.89	53.4	17.5	34.6	23.0	19.6	18.3	27.1	19.4	97.6	2.37	1.84	
68	127	78	27.5	19.2	2.81	2.25	177.4	87.4	62.2	77.3	38.2	68.7	43.4	55.9	61.3	62.7	1.93	1.68
273*	112	60	50.5	40.7	2.48	1.18	140.2	29.8	86.6	25.7	56.6	32.2	62.4	40.0	155.2	2.87	2.07	
89	117	81	24.8	27.8	2.05	1.67	117.8	73.7	92.3	99.3	40.2	58.1	59.6	97.1	173.3	4.18	2.92	
															140.5	48.4	1.78	1.42

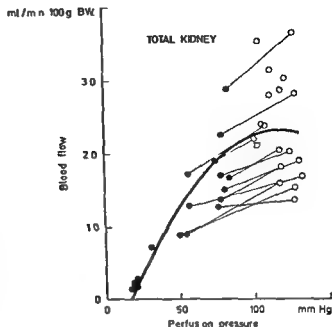


Fig. 2a. Autoregulation of blood flow in the total kidney. The open circles represent the pre-clamped values and the filled circles the clamped values.

relation  $y = a_0 + a_1x + a_2x^2$  as calculated with the least square method. The blood flow-pressure relationship of the superficial cortical zone seems not to deviate from a linear relation while the curves of zones 2, 3 and 4 show an autoregulatory pattern. In the inner zones there is a tendency to an increased blood flow at a moderate reduction in the perfusion pressure. Utilizing all data from zones 3 and 4 this increase was shown to be significant ( $0.01 < P < 0.02$ ). The values of the single glomerular blood perfusion rates show quite the same picture as the corresponding zones. In Fig. 3 the fractional blood flow in the cortical zones are related to the arterial blood pressure and it is seen that a reduction in the arterial blood pressure from the control values to about 70 mm Hg resulted in a redistribution of the blood flow. A relative decrease is clearly seen in zone 1 and concomitant increases are observed in zones 3 and 4 while zone 2 was unchanged. This distribution remained essentially unchanged by a further reduction in the perfusion pressure.

In Fig. 4 the percentage change in cardiac output, total and regional blood flow in the right control kidney are depicted. The figure shows that the experimental manoeuvre in itself will be concomitant with a moderate reduction of these blood flows in parallel with a reduction in the cardiac output.

#### *Micropuncture data*

The hydrostatic pressures within the superficial cortical vasculature and the proximal III tubules are depicted in Table II. The pressures in the glomerular capillaries and the welling points are shown in Fig. 5. From this figure it would seem that both the glomerular capillary pressure and the welling point pressure are autoregulated. A similar relation is also found for the peritubular capillary pressures as well as the proximal tubular pressures. For

TABLE II Hydrostatic pressures within the superficial cortical vasculature and the proximal tubules of different arterial perfusion pressures

Rat Weight g	Condi- tion	Perfusion pressure mm Hg	Glom capl pressure mm Hg	Welling point pressure mm Hg	Peritub capl pressure mm Hg	Tubular pressure mm Hg	Int filtrat. pressure mm Hg	Pressure drop aff arteriole mm Hg	Pressure drop eff arteriole mm Hg
1 286	control	118	68.0	13.0	12.0	15.0	31.0	50	55
	clamp	81	53.0	13.0	9.0	13.3	17.7	28	40
	control	132	61.0	15.0	—	14.6	24.4	71	46
	clamp	108	59.0	14.0	10.0	12.8	24.2	49	45
	clamp*	73	48.0	—	—	11.8	14.2	25	—
2 298	control	114	56.6	10.5	—	11.5	23.1	57.4	46.1
	clamp	72	46.8	12.3	11.5	11.8	13.0	25.2	34.5
	control	127	64.0	18.0	—	19.3	22.7	63.0	46.0
3 280	control	118	55.5	13.3	9.3	11.5	22.0	62.5	42.2
	clamp	81	48.1	12.9	8.5	11.8	14.5	32.9	35.2
	control	106	54.2	12.8	9.5	12.1	20.1	51.8	41.4
	clamp	65	40.5	13.5	9.3	11.3	7.2	24.5	27.0
4 290	control	118	53.9	14.1	9.5	10.1	21.8	64.1	39.8
	clamp	79	42.1	12.5	6.5	9.4	10.7	36.9	29.6
	clamp	69	40.5	8.0	—	9.8	8.7	28.5	37.5
	clamp	55	31.0	10.0	—	6.0	3.0	24.0	21.0
5 310	control	115	52.0	15.0	11.3	9.4	20.6	63.0	37.0
	clamp	76	42.0	13.5	7.5	9.8	10.2	34.0	28.5
	control	100	55.2	—	10.0	10.5	22.8	—	—
	clamp	56	38.9	12.2	9.5	10.2	6.7	17.1	26.7
6 260	control	118	57.7	13.3	9.5	12.3	21.4	60.3	44.2
	clamp	76	50.7	11.5	7.3	10.7	18.3	25.3	39.2
	clamp	26	—	4	—	—	—	—	—
±SD	control	118±7.5	58±5.3	14±2.1	10±1.2	13±3.0	23±3.3	60±6.5	44±5.1
	clamp	74±14.4	45±7.7	12±1.8	9±1.5	11±2.0	12±6.1	30±8.5	33±7.0

rat kidney and the results are contradictory. Thus Ohler *et al* (1959) did not find any autoregulation while others did (Weiss *et al* 1959). It might not be excluded that the anaesthesia influences on the ability of autoregulation.

According to Table I and Fig. 4 there is a reduction in the cardiac output when the aorta is clamped between the two renal arteries. This reduction in the cardiac output was concomitant with a decrease in the total blood flow of the control kidney to the same extent but with apparently no redistribution in the cortical blood flow indicating a generalized increase in the renal vascular resistance. From the present data nothing can be said regarding the location of that resistance change.

The autoregulatory properties of the various parts of the renal parenchyma differ according to some authors (Thurau *et al* 1960) who found autoregulation weak or absent in the medulla. Other authors, however, have not found any differences between the cortical and the medullary regions (Aukland 1966, Wolgast 1968, Grängsjö and Wolgast 1972, Löyning 1971). On the other hand, the results of this investigation indicate large differences in the autoregulatory power of the inner and outer cortex. The outer cortical zone did not show any autoregulation of the blood flow. Zone 2 showed a slight autoregulation, zones 3 and

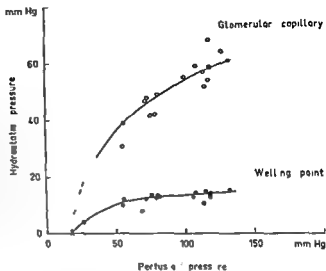


Fig. 5 The picture shows the hydrostatic pressures in the welling points at different perfusion pressures. The calculated values of glomerular capillary pressures are also represented in the figure. The glomerular capillary pressure was calculated by adding 22 mm Hg (plasma oncotic pressure) to the stop flow pressures measured.

4 did not only show an autoregulation but also an increased blood flow at a moderately decreased perfusion pressure. These results agree well with those obtained by McNay and Abe (1970) who used the microsphere technique on dog kidneys. The results of both these investigations indicate a vasoactive response to a decreased perfusion pressure which must be regarded as remarkable. The heterogeneity of the vasoreactivity in the different zones of the cortex leads to a marked redistribution of the blood flow in favour of the inner parts of the cortex and also the medulla (Fig. 3) through the true juxtamedullary glomeruli.

In other investigations dealing with the autoregulatory problems the arterial blood pressure was increased by clamping the carotids with or without cutting the vagal nerves. With this technique the arterial blood pressure and the renal perfusion pressure are increased but during the manoeuvre the sympathetic outflow may also be increased and it is plausible to assume an increased tone in the vasoconstrictor nerves to the kidneys. The experimental situation thus implies both a vasoconstriction in the kidney and an increased perfusion pressure. This is not comparable with the condition analyzed here. In experiments on haemorrhagic shock, Hellberg *et al.* (1973) found a linear relation between the cardiac out

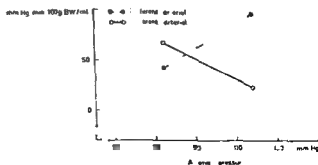


Fig. 6 The figure summarizes the resistance of the single afferent and efferent arterioles before the clamp and during the clamping.



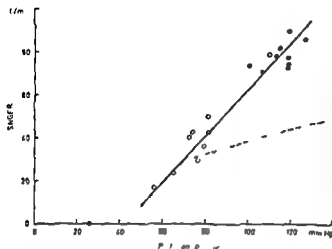


Fig 7 Single nephron glomerular filtration rate (SNGFR) versus the arterial perfusion pressure. SNGFR is a calculated value according to the text. The filled circles represent control normotensive conditions and the unfilled circles the values during the clamping. The dashed line represents the SNGFR when an equilibrium does not exist at the end of the glomerular capillaries and is obtained according to the text.

put and the total and the regional renal blood flow indicating a vasoactive response quantitatively the same in all parts of the cortical parenchyma—which is in agreement with the present findings (Fig 4).

The mechanisms behind the change in blood flow distribution in the autoregulating kidneys were analyzed in experiments where the hydrostatic pressures in the vascular structures on the renal surface were measured and related to the blood flow determined with the microsphere technique. The results were very homogeneous and were regarded as being representative for nephrons having their glomeruli in the outer part of the cortex. The results are not in accordance with the common opinion that the vasoactive response is restricted to the afferent arterioles but indicate that both the arterioles will be involved. This opinion was also reached by Robertson *et al* (1972) using essentially the same approach.

In 1966 Gertz *et al* measured the glomerular capillary pressure with the intratubular stop flow pressure technique and obtained glomerular capillary pressures within the range 85–90 mm Hg which values are very high when compared with the pressures of some 60 mm Hg found in this study. The difference in the results depend probably on the fact that Landis' technique used by Gertz *et al* has a tendency to give too high pressures. Of interest is that their results showed an autoregulatory tendency in the glomerular capillary pressure in a similar way as found in this investigation. Their constant capillary pressure in the hypertensive experiments must however partly be excluded from the discussion of autoregulatory mechanisms because of the dubious effect of carotid clamping—as discussed earlier.

By multiplying plasma flow with a filtration fraction obtained from the effective filtration pressure (Landis and Pappenheimer 1963) a measure of the glomerular filtration rate in the superficial region can be calculated. The hematocrit was assumed to be 50%, and the protein concentration of plasma 6 g/dl. A complete filtration equilibrium (i.e. the net driving forces equal zero) is regarded to exist at the end of the glomerular capillary (Brenner 1971). The SNGFR of the normotensive animals were according to Fig 7 calculated to be about 80 nl/min. This value is reasonably too high in comparison with direct measurements and the

discrepancy between the calculated and expected values seems most likely to depend on the erroneous assumption of a filtration equilibrium at the end of the glomerular capillary. In a forthcoming paper (Källskog *et al* 1975) direct measurements of the glomerular filtration rate and the single glomerular plasma flow are presented and the existence of a disequilibrium is predicted. Values from that study would give an autoregulation of the SNGFR, shown in Fig. 7 as the dashed line.

The physiological significance of the different autoregulatory properties of the glomerular populations is somewhat unclear. Barger (1973) has reviewed the literature and made the assumption of outer salt losing nephrons and inner salt saving nephrons. The first indications of such a possibility were suggested by Horster and Thurnau (1968) and Gertz *et al* (1969) in investigations showing a rearrangement of the glomerular filtration. At high sodium intake the GFR rose in the outer glomeruli and decreased in the inner ones while the opposite happened at low sodium intake. The diversion of blood from the inner salt saving nephrons to the outer salt losing nephrons when the blood pressure is increased might then explain the nearly linear relationship between the arterial blood pressure and the urine flow at an arterial blood pressure above some 50 mm Hg.

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## A Theoretical Study of Restricted Convection-Diffusion as Applied to Blood-Tissue Barrier Exchange

By

BO ÅBERG

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### Abstract

ÅBERG B *A theoretical study of restricted convection-diffusion as applied to blood-tissue barrier exchange* Acta physiol scand 1975 94 301-308

This paper handles a model of the capillary function in the exchange of uncharged molecules between the blood and the tissue. The capillary system is subdivided into a filtering and a reabsorbing part. The exchange is assumed to occur through channels which are described in operational terms as pores. Through these pores there is a transport of solutes by concomitantly acting convection and diffusion influenced by a steric hinderance (restricted convection-diffusion). The outflux of glucose and raffinose is enhanced in the filtering pores, raffinose relatively more than glucose. In the reabsorbing pores the outward diffusion is hindered to some extent, raffinose relatively more than glucose. It is shown that the net effect of filtration and reabsorption is to increase the outflux of raffinose as compared to that of glucose. This mechanism may explain why glucose and raffinose and other small molecules appear to pass across capillary walls in proportion to their free diffusion coefficients and not in proportion to their restricted diffusion coefficients.

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The transport of neutral solutes across capillary walls is generally assumed to occur through pores (opened intercellular junctions cf Karnovsky 1970). In experiments where the plasma concentration of dextran molecules of different sizes were held constant and plasma and lymph concentrations were compared, the average pore radius could be estimated to about 4 nm (cf Grotte 1956). The results indicated that low molecular weight dextrans passed out of the capillaries in proportion to their restricted diffusion coefficients. A restriction due to steric hinderance should also affect the outflux of such molecules as glucose and raffinose through the capillary wall (Beck and Schultz 1970) but such a restriction could not be found in *in vivo* experiments with single injection technique (Crone 1963, Kampp 1970). In a series of *in vitro* experiments with rather high precision it could be verified that the outflux of raffinose and glucose indeed seemed to occur without restriction due to steric hinderance in pores (Åberg 1974).

In his model of the capillary system Starling (1896) proposed that there is filtration of fluid in the arterial part of the capillary and reabsorption in the venous part. A fluid filtration may oppose the hinderance exerted by steric restriction in a single pore (Åberg

Hägglund 1974) The aim of this communication was to evaluate if simultaneous convection and diffusion may give outflux rates of low molecular weight solutes in single-injection experiments that are proportional to the free diffusion coefficients rather than to the restricted diffusion coefficients

## The Model

### Restricted convection diffusion

The transport of neutral molecules through pores in the capillary wall is considered to occur by concomitant diffusion and convection. The word pore is here used as an operational term. If  $J_p$  is the solute flux density,  $c(x)$  is the concentration along inside of the pore,  $v$  the mean linear fluid flow velocity, and  $D$  the free diffusion coefficient, then the flux density (mass flow velocity per unit pore area) is calculated as

$$J_p = -k \frac{D}{\partial x} c(x) + k c(x) \quad (1)$$

where  $k$  is a conductance coefficient assumed to be valid for both diffusional and convectational transport. This coefficient is equal to the ratio between the apparent pore area per 100 g tissue ( $A$ ) for the solute and the pore area per 100 g tissue ( $A_p$ ). It has generally been related to the molecular and the pore dimensions (Bean 1972)

$$k = A/A_p = [2(1-a/r)^3 - (1-a/r)^6] g(a/r) \quad (2a)$$

$$g(a/r) = 1 - 2a^2/3r^3 - 0.162(a/r)^3 - 0.406(a/r)^7$$

where  $a$  = molecular radius and  $r$  = pore radius

The free diffusion coefficient  $D$  is assumed to be satisfied by the Stokes-Einstein relation

$$D = RT/(6\pi \eta N a) \quad (3)$$

where  $R$  is the gas constant,  $T$  is absolute temperature,  $\eta$  is viscosity and  $N$  is Avogadro's number

With proper choice of test substances the conditions within the pore can be approximated by a steady state transport (build up time of the concentration profile is short as compared to transport time Åberg and Hägglund 1974, Åberg 1974). Eq. (1) is then solved by integration, putting  $c(x) = c_0$  for  $x=0$  (plasma side) and  $c(x) = c_d$  for  $x=d$  (tissue side)

$$J_p = k \frac{c_0 - c_d \exp(-v d/D)}{1 - \exp(-v d/D)} \quad (4)$$

The concentration profile within the pore is unaffected by  $k$ , as  $k$  is assumed to have the same influence on both the convectational and the diffusional transport (cf. Forster 1971)

### Model of the capillary

The capillary system is subdivided in two main parts: one with filtrative and the other with reabsorptive properties. In the filtering or arterial part of the capillary system the total pore area per 100 g tissue is  $A_{p1}$ , the linear flow velocity through the pores  $v_1$ , and the corresponding solute flow  $J_1 A_{p1}$ . In the reabsorbing part the total pore area per 100 g tissue is  $A_{p2}$ , the linear bulk flow through the pores  $v_2$ , and the solute flow  $J_2 A_{p2}$ —all parameters assumed operational model averages

In the general case the solute balance is given by

$$J_1 A_{p1} + J_2 A_{p2} - L c_d = 0 \quad (5)$$

where  $L$  = lymph volume flow. The corresponding fluid balance is governed by

$$v_1 A_{p1} + v_2 A_{p2} + F - L = 0 \quad (6)$$

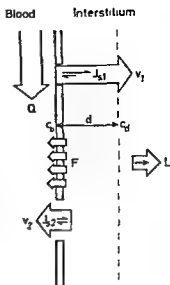


Fig. 1 Model of the capillary. The capillary system is reduced to a single capillary with filtering and reabsorbing parts. In the filtering part the linear velocity of the fluid flow is denoted as  $v_1$  and in the reabsorbing part as  $v_2$ . In constant concentration experiments the capillary and tissue fluid concentrations are assumed to be in balance. When the concentration on the tissue side of the transport path  $d$  is zero (single injection type of experiments) there is a net flux of solute from the capillary to the tissue ( $J_1$  and  $J_2$ ).  $Q$  is blood flow,  $L$  lymph flow and  $F$  is flow of fluid free from the test solute.

where  $F$  is the reabsorption of fluid free from the test solute (Fig. 1).  $F$  is introduced mainly to balance (6). It may be seen as a solvent flow from the tissue to the blood through small pores or through the endothelial cells not available to a particular solute as discussed by Yudkevich and Alvarez (1967), Ussing (1970) and Tosteson (1970).

#### Application of the convection-diffusion model to single injection technique

In the single injection technique the transport of solutes across the blood-tissue barrier is expressed in terms of extraction ( $E$ ). This is a measure of the loss of solute into the tissue as compared to the amount of solute injected into the capillary. As the solute loss is solute flux density times transport area and amount injected is plasma concentration times plasma flow, a general formulation of extraction is

$$E = \frac{A_p J(t)}{Q c(t)} \quad (7)$$

where  $Q$  is plasma flow per 100 g tissue and  $c(t)$  the concentration in the capillary.

The solute flow  $J(t)$  is proportional to the momentary concentration  $c_d(t)$  (Åberg and Hägglund 1974) and the extraction is consequently time invariant

$$E = \frac{A_p J}{Q c} \quad (7a)$$

In the situation in which the tissue concentration  $c_d = 0$  the following equation is obtained from Eqs (4) and (7a)

$$E = \frac{k_1 F A}{Q \{1 - \exp(-v d/D)\}} \quad (8)$$

where  $E$  is the extraction in the arterial part of the capillaries and  $k_1$  is the conductance factor (Eq. 2) in the filtering pores. In the venous part, the plasma concentration of the solute is  $(1 - E) c_0$ . The passage of solute within the capillary is assumed fast as compared to the longitudinal tissue diffusion. The tissue concentration is accordingly still assumed to be zero. This assumption is justified if the extraction is during the passage of the major part of the bolus (Åberg 1974). The venous extraction  $E_v$  is then by

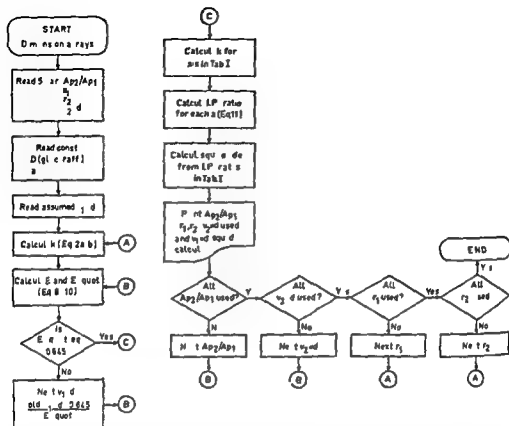


Fig. 2 Flow diagram of calculations. The variables are inserted in arrays of 5 members. Each possible combination of the members is used for calculation of the 5th variable  $\alpha$  d (left column). With the aid of all five variables the function of the lymph to plasma concentration ratio (LP ratio) against the diffusion radius ( $\alpha$ ) is calculated and compared by the sum of square deviations to the experimental values of Grotts (1956). When all 625 results are obtained the minima of the square deviations are studied by new sets of variables with smaller increments.

$$E_1 = \frac{k_1 \cdot v_1 \cdot A_{ps} \cdot (1 - E_1)}{Q \{1 - \exp(-v_1 d/D)\}} \quad (9)$$

where  $k_1$  is the conductance factor in the reabsorbing pores. Now the extraction may be assumed to occur first in the filtering capillaries and subsequently in the reabsorbing capillaries. The combined extraction  $E$  is then

$$E = E_1 + E_2 \quad (10)$$

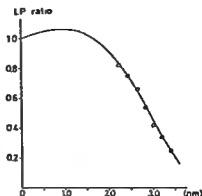
*Application of the restricted convection diffusion model to the constant concentration technique*

In the constant concentration technique the permeability characteristics are derived from the LP ratios i.e. lymph (or tissue fluid) concentration ( $c_d$ ) over plasma concentration ( $c_p$ ).  $c_d/c_p$  may be solved in terms of the transport parameters by combining Eqs (4) and (5)

$$\frac{c_d}{c_p} = \frac{k_1 \cdot v_1 \cdot A_{ps} / (1 - p) + k_2 \cdot v_2 \cdot A_{ps} / (1 - q)}{k_1 \cdot v_1 \cdot A_{ps} / p / (1 - p) + k_2 \cdot v_2 \cdot A_{ps} / q / (1 - q) + L} \quad (11)$$

where  $p = \exp(-v_1 d/D)$  and  $q = \exp(-v_2 d/D)$

Fig 3 Best fitted parameters Lymph to plasma concentration ratio (LP ratio) for different molecular radius ( $a$ ) theoretically with the best fitted values of the parameters in Tab I (continuous line) compared to the experimentally found values (circles)



### General assumptions Numerical Calculations from Model Concepts

In order to obtain a rough estimation of the transport parameters involved, the data from single injection experiments by Åberg (1974) and continuous infusion experiments by Grotte (1956) are used. The total pore area ( $A_{p1} + A_{p2}$ ) is assumed to be  $1.4 \text{ cm}^2/100 \text{ g tissue}$  (Karnovsky 1970) and the transport path length  $d$  is set to  $10 \text{ } \mu\text{m}$  which includes an equivalent path through unstirred layers.

Albumin is known to pass the capillary walls with difficulty. Assuming reabsorption at the venous end of the capillaries which probably is more permeable than the arterial end (Smith and Rous 1931; Hauck and Schroer 1969) although still not permeable to proteins to a visible extent (Wiederhielm 1968) the operational reabsorption pore radius ought not to be larger than about  $6 \text{ nm}$ . Values between  $4$  and  $6.5 \text{ nm}$  are tested in the model.

The extraction quotient raffinose over glucose is set equivalent to the ratio of their free diffusion coefficients  $0.645$ . The remaining parameters  $A_{p2}/A_{p1}$ ,  $r_1$ ,  $r_2$ ,  $r_3$  and  $r$  are solved in a trial and error procedure in the following manner (Fig. 2).  $A_{p2}/A_{p1}$ ,  $r_1$  and  $r_2$  are five values of each are inserted into Eqs (8)–(10) whereby 625 values of  $r_3$  and  $d$  are obtained each value corresponding to one value of the other parameters. Those sets of values which fit best to the 6 LP ratios of Grotte inserted to Eq (11) are then varied within smaller limits again in Eqs (8)–(10) until the best possible visual fit is obtained. The difference of  $A_{p2}$  and  $r_3$  is a flow of water in routes other than through pores (lymph and through endothelial cells or "tight junctions") here noted  $L + F$ .

This fitting shows that if  $A_{p2}/A_{p1}$  is too small i.e. reabsorption through pores is small the reabsorption through cells becomes large and the extravascular molecules will be too much concentrated in constant concentration experiments. The same will be the case if the reabsorption flow  $r$  is too small. The effect of varying  $r_1$  and  $r_2$  of course is to set the limits for the passage of the molecules.

### Results

The results of the mathematical procedure in order to correlate the model to the experimental data are shown in Fig. 3 where the results of the final best visual fit was obtained. The best fitted set of parameters are listed in Table I.

The influence of the parameters on the model behaviour is shown by varying them  $\pm 10\%$ .



TABLE 1 Input to and output from the numerical calculations on the model

## Constants used

## 1 Single Injection situation

Mean plasma flow = 0.178 ml/s

Mean tissue weight = 230 g

Extraction ratio raffinose/glucose = diffusion coeff. ratio = 0.645

## 2 Constant concentration situation (Grotte 1957)

Diffusion radius (nm) 2.22 2.42 2.64 2.83 3.01 3.20

L/P ratio 0.82 0.75 0.66 0.54 0.42 0.34

Mean lymph flow (L) =  $7 \cdot 10^{-6}$  ml/s and 100 g tissue (estimated)

## Best fitted variables to the constants used

 $v_1 d = 0.26 \cdot 10^{-6} \text{ cm}^2/\text{s}$  $v_2 d = -0.16 \cdot 10^{-6} \text{ cm}^2/\text{s}$  $r_1 = 3.9 \text{ nm}$  $r_2 = 5.1 \text{ nm}$  $d = 5 \mu\text{m}$  $A_{p2}/A_{p1} = 1.15$ 

keeping the others constant at their calculated values. The result is shown in fig. 4a-c. This model is relatively more sensitive to parameter variations in the constant concentration situation (continuous lines) than in the transient concentration (single injection) situation with smaller molecules (figures in the upper right corners). It is most sensitive to variations in the pore diameters, and for that reason these parameters seem to be those which are best determined.

## Discussion

## The magnitude of the parameters

A calculation of the filtration flow velocities from a measure of the relative movement of red blood cells with the microocclusion technique has been done by Zwiefach and Intaglietta (1966). The fluid movement from the capillary to the tissue ranged from 0.119 to  $0.070 \mu^2 \mu^{-2} \text{ sec}^{-1}$ . Assuming again that 0.02 per cent of the vessel area consists of opened intercellular junctions and that  $d$  is  $5 \mu\text{m}$ , the values of Zwiefach and Intaglietta can be recalculated to a  $v_1 d$  of  $3 \cdot 10^{-6}$  to  $0.5 \cdot 10^{-6} \text{ cm}^2/\text{sec}$ , which very much exceeds the values of  $0.26 \cdot 10^{-6}$  to  $0.16 \cdot 10^{-6} \text{ cm}^2/\text{sec}$  calculated in this work.

The sum of forces needed to exert a fluid filtration velocity times diffusion path length

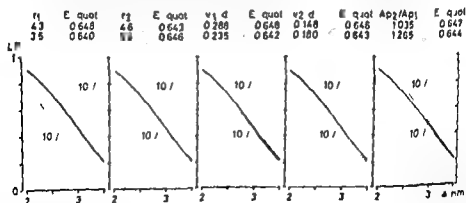


Fig. 4 Parameter variations. The effect of a 10% variation of one of the parameters in 1. (1)  $r_1$  and  $r_2$  are given in nm.  $v_1 d$  and  $v_2 d$  in  $\text{cm}^2/\text{sec} \cdot 10^6$ . The effect on extraction quotients (E quot) are small. The model is most sensitive to variations in pore radius.

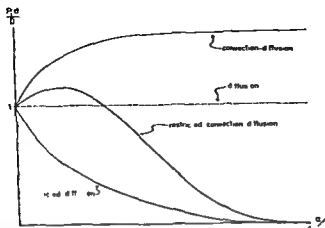


Fig 5 Comparison between different transport mechanisms. The permeability of a substance ( $P_d$ ) per its free diffusion coefficient ( $D_f$ ) per transport distance ( $d$ ) is constant in unrestricted diffusion. In convection-diffusion it reaches a plateau where the diffusional part is negligible (has no meaning here as transport is unrestricted  $a/r$  is to be seen as a molecular dimension only). In restricted diffusion the permeability falls with increasing ratio of the molecular ( $a$ ) to the pore ( $r$ ) radius. In restricted convection-diffusion the permeability increases in constant, or decreases depending on the convection flow velocity and the ratio  $a/r$ .

of  $0.26 \cdot 10^{-4}$  cm<sup>2</sup>/sec through a pore with a radius of 3.9 nm is hard to evaluate. If the law of Poiseuille was valid the force needed should be about 3.5–4 kPa (25–30 mm Hg) if the thickness of the capillary wall is about  $0.5 \mu\text{m}$  but if the restriction is represented by a short distance along the pore the force needed for water propagation is less and within reasonable values for the transmural pressure in the arterial parts of the capillary (The constriction effect Perl 1971).

On the other hand the flow velocity might be underestimated. Restriction is calculated according to an empirical formulation emanating from works by Faxén (1922) and Ferry (1936). Renkin has used this restriction formulation in a somewhat different way (Renkin 1954) and if his formulation is used the filtration flow velocity must be increased about 5 fold. As an intermediate Verniory *et al* (1973) used different formulations for restriction of the molecules if they were subjected to a chemical or a velocity gradient across the glomerular wall. In those cases the ordinary Starling forces might not be sufficient.

### Concluding remarks

In single injection experiments smaller molecules are transported across the walls of the muscular capillaries as if there was no restriction whereas in constant concentration experiments the restriction of larger molecules is obvious. In Fig 5 the general behaviour of restricted convection-diffusion transport is described in comparison to diffusion and convection-diffusion without restriction and the restricted diffusion in terms of permeability per transport path length for different molecular sizes. Transport by a restricted diffusion should be substantially hampered already at small ratios of molecule/pore radius which seems not to be the case. Restricted convection-diffusion meets with no apparent restriction to smaller molecules but with restriction to the larger ones. The length of the apparently

unrestricted part (LP ratio about unity in Fig. 3 permeability equal to or larger than  $D/d$  in Fig. 5) and the slope of restriction can be varied by the model parameters. The fitting of this model to experimental results can be done with reasonable values of these parameters.

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## The Effect of Restricted Convection-Diffusion on Bolus Concentration along an Exchange Vessel

By

BO ÅBERG

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### Abstract

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ÅBERG B *The effect of restricted convection-diffusion on bolus concentration along an exchange vessel* Acta physiol scand 1975 94 309-312

If a sudden rise in solute concentration travels through a capillary system a diffusible neutral solute passes the walls of the exchange vessels. If then the rate of passage depends on a chemical potential gradient (diffusion) and a hydrodynamical potential gradient (convection) and is sterically restricted (restricted convection-diffusion) water and low molecular weight solvents leave the filtering part of the capillary at a higher rate than the solute causing a relative increase in its concentration. This phenomenon and the effect of an increasing pore size along the capillary is investigated in a model for an impermeable solute and two diffusible solutes corresponding to glucose and raffinose. It is shown that in the model the concentration fall along the capillary tends to become rectilinear.

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The transport of uncharged molecules across capillary walls might be calculated from the disappearance of a bolus injected into the capillary system. In this case a longitudinal intra-capillary concentration profile of the substance must be assumed. The most often used assumption is that the concentration falls exponentially along the exchange vessel (Crone 1963, Goresky *et al.* 1970). This assumption is valid if there is no flow of water across the blood-tissue barrier and if the permeability is constant along the capillary, but this does not seem to be the case (*cf.* Hauck 1969).

In an earlier work (Åberg 1974b) it has been pointed out, that the chemical potential can not alone give rise to the experimentally found transport rates of smaller molecules across the capillary wall if the transport is considered to occur through sterically restricting pores. If there is however an additional convection transport the effect of restriction on the smaller molecules can be counteracted.

The introduction of a filtration and a reabsorption as well as a gradient of permeability along the capillary affects the concentration along this vessel. The aim of this communication was to study the concentration decrease along an exchange vessel in a simple mathematical model having larger pores towards the venous side with a filtration from lumen to the tissue on the arterial side and a reabsorption on the venous side.

## Method

The calculation was made with a finite difference method. The capillary length was divided in sections; the concentration in each section calculated as a result of water and solute flows across the wall and the blood flow and the concentration in the preceding section.

## Assumptions

The pore radius ( $r$ ) is assumed to increase linearly along the length ( $l$ ) from entrance to exit of the exchange vessel.

$$r(l) = r(0) + k \cdot l \quad (1)$$

where  $k$  is a constant.

The convective flow along the transport distance ( $d$ ) is assumed to depend on pore radius ( $r(l)$ ) and a net driving force. The driving force tends to be relatively complex even in a simple model like this. The pressure drop along a channel with porous walls is not rectilinear but larger in the beginning than at the end even if the wall porosity is homogeneous (Herman 1953). Also the osmotic force might decrease in a non-rectilinear fashion depending on the decreasing reflection coefficient for the permeant molecules. For the sake of simplicity the net driving force is assumed to fall exponentially along the capillary. The force is positive in the filtering parts, negative in the reabsorbing parts and zero at the point of balance.

$$v(l) d = v(0) d \{ \exp(-k_1 l) - \exp(-k_2 l_0) \} / \{ v(0) - k_1 v(0) \} \quad (2)$$

where  $k$  is a constant.

The solute flux ( $J_s$ ) across each section is assumed to occur by convection-diffusion (Albert 1974a). The intracapillary concentration is assumed to be partially homogeneous ( $c$ ) and the concentration on the tissue side of the transport distance ( $d$ ) is assumed to be zero (1) although it is linear over the transport distance in the case of filtration and reabsorption (Albert 1974a).

$$J_s(l) = v(l) \Delta p \cdot q(l) + (D/d) [c(l) - 0] \quad (3)$$

where  $\Delta p$  is the pore area of each section,  $D$  is the free diffusion coefficient and  $q$  is a confluence factor related to the pore radius ( $r$ ) and the molecule radius ( $a$ ).

$$q(l) = [2(1 - b) + (1 - b)^2] / [1 + 2(1 - b)^2 + 0.474b^2] \quad (4)$$

$$\text{where } b = a/r(l)$$

## Numerical calculations

The model parameters are given in Table 1, obtained from earlier calculations (Albert 1974b). The vessel is divided into 43 sections of which 20 are filtering and 23 are reabsorbing (Reabsorbing area/filtering area = 1.15). At the entrance of the exchange vessel the concentration is set to unity ( $c(0) = 1$ ).

Table 1. Input parameters to the model. The magnitudes of the parameters are the best fitted to a restricted convective flow model taken from an earlier work (Albert 1973).

Mean filtration flow velocity transport distance—	0.26 $10^{-6}$ cm/s
Mean reabsorption flow velocity transport distance—	0.16 $10^{-6}$ cm/s
Mean filtering pore radius—	2.7 nm
Mean reabsorbing pore radius—	3.1 nm
Plasma flow (l/min) g tissue—	0.075 cm/s
Plasma filtering reabsorbing area—	1.15

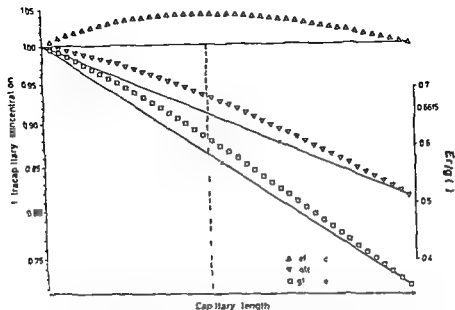


Fig. 1 Results of calculations. The exchange capillary is divided into 43 sections and the concentration of a passing bolus is calculated in each section for a reference substance and raffinose and glucose as a result of restricted convection-diffusion transport across the capillary wall. The concentrations are plotted on a logarithmic scale (left scale) and the build up of an extract quotient raffinose/glucose ( $Er/g$ ) can be followed (right scale).

Assume  $i$  and  $j$  to be two consecutive sections.  $Q$  is the plasma flow and  $\Delta F$  is the trans-endothelial flow free from the solute investigated in each section. Then

$$Q(i) = Q(i) - r(i) \times \Delta p - \Delta F \quad (5)$$

$$C(i) = [Q(i) \times C(i) - J(i)]/Q(i) \quad (6)$$

From Table I  $h$  and  $r(o)$  are easily calculated to 0.0558 nm/l and 3.342 nm respectively.  $\Delta p$  was set to  $1.4/43 = 0.0326$  cm<sup>2</sup>/100 g tissue (Total capillary area = 7.000 cm<sup>2</sup>/100 g tissue whereof 0.02% consists of opened intercellular junctions Karnovsky 1970) and  $d$  was set to 5  $\mu$ m.

$\lambda$  and  $r(o)$   $d$  has been calculated by iterations from Eq. (2) so that  $[\sum_{i=1}^n r(i) \cdot d]/20 \sim 0.26$  10<sup>-3</sup> cm/sec and  $[\sum_{i=1}^n r(i) \cdot d]/23 = -0.16$  10<sup>-3</sup> cm<sup>2</sup>/sec.

### Results

The results from the calculations of 3 different molecules are shown (Fig. 1) one ideal reference ( $a \sim 10$  nm) and two diffusible molecules ( $D = 0.55 \cdot 10^{-6}$  cm<sup>2</sup>/s  $a = 0.61$  nm corresponding to raffinose and  $D = 0.85 \cdot 10^{-6}$  cm<sup>2</sup>/s  $a = 0.39$  nm corresponding to glucose). The exponential concentration fall is represented by a straight line. The concentration fall of glucose happens to be nearly rectilinear and that of raffinose is more convex.

### Discussion

A bolus of neutral solutes is supposed to pass an exchange vessel. When there is a convective flow of water to the tissue and the solute transport across the capillary wall is relatively more restricted than the water transport, the solutes remaining inside the capillary are more concentrated than without that convection flow. Calculating with an exponential fall in the concentration would in this case give rise to a slight underestimation of the chemical potential gradient across the blood-tissue barrier.

In an earlier work (Åberg 1974 b) the transport calculations were simplified by taking the arterial concentration as the chemical driving force to the balance point  $l_p$  from where the corresponding force was set equal to the venous concentration. This calculation in steps obviously led to an overestimation of the arterial driving force and an underestimation of the venous driving force. The combined effect of this can be evaluated from the present results. Starting with an extraction quotient of 0.645 the parameters used in the present calculations were estimated. Here the calculations are made "the other way around" which leads to about the same extraction ratio 0.662.

In conclusion a flow of water from the exchange vessels to the tissue has a twofold increasing effect on the solute transport: (a) to give an additional convective transport from the blood to the tissue and (b) to increase the chemical activity of solutes more restricted than water.

I wish to express my thanks to C. A. Goresky for initiating this work.

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## Enzyme Activities and Muscle Strength after "Sprint Training" in Man

By

A THORSTENSSON, B SJÖDIN and J KARLSSON

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### Abstract

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THORSTENSSON A, B SJÖDIN and J KARLSSON *Enzyme activities and muscle strength after sprint training in man* Acta physiol scand 1975 94 313-318

Sprint type strength training was performed 3-4 times a week for 8 weeks by 4 healthy male students (16-18 yrs). The training was carried out on a treadmill at high speed and with high inclination. Muscle biopsies were obtained from vastus lateralis before and after the training period for histochemical classification of slow and fast twitch muscle fibres and for biochemical determination of metabolites and enzyme activities. Muscle fibre type distribution was unchanged whereas fibre area indicated an increase for both fibre types in 3 subjects after training. The muscle enzyme activities of  $Mg^{2+}$  stimulated ATPase, myokinase and creatine phosphokinase increased 30, 70 and 36 percent, respectively. Muscle concentration of ATP and creatine phosphate (CP) did not change with training. Sargent's jump increased with on average 4 cm (from 47 to 51 cm), maximal voluntary contraction (MVC) with 19 kp (from 165 to 184 kp) and endurance at 50 percent of MVC with 9 s (from 47 to 56 s), respectively. After training all subjects showed a gain in body weight (mean 1.4 kg) and in thigh circumference (mean 1.5 cm) indicating a larger leg muscle volume and consequently also an increase in total ATP and CP.

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High intensity exercise of short duration will apply high demands on the immediate energy supplying mechanisms in the muscle and repeated exercise of this type has been shown to cause exhaustion of phosphagen stores in the exercising muscles (Karlsson and Saltin 1971). Animal studies have demonstrated that short term high intensity training programs cause an increase in creatine phosphokinase activity (Staudte, Exner and Pette 1973) and an increased storage of creatine phosphate has been indicated after sprint training of rats at high altitude (Gale and Nagle 1971).

The aim of the present study was to investigate quantitatively and qualitatively the effects of a high intensity intermittent sprint training program on skeletal muscle in man. Special interest was focused on the turn-over mechanisms of ATP by examining the activities of actomyosin ATPase (E.C.3.6.1.3), creatine phosphokinase CPK (E.C.2.7.3.2) and myokinase MK (E.C.2.7.4.3) as well as the size of the phosphagen stores before and after the training period. In addition histological analyses were performed to evaluate changes in muscle fibre type composition.



## Material and Methods

**Subjects** 4 healthy moderately trained male students (16–18 yrs) participated in the study. Although the subjects were in late adolescence, growth effects are assumed to be insignificant due to the short observation period. No increase in body height was observed during the period.

**Training procedure** The subjects were training 3 to 4 times a week over a period of 8 weeks. The training bouts were performed as short term intermittent sprint training (3 s running) on a motor-driven treadmill at high speed and with steep inclination. The rest periods between each bout were 25–55 s. Over the training period the speed, inclination and number of repetitions were successively increased from on average 19 km/h, 9° and 20 repetitions per bout to 24 km/h, 10° and 40 repetitions per bout. The basis for these gradual increases in training load and intensity was to force the subjects to approximately the same level of exhaustion after each training session as the training program proceeded and the capability of the subjects improved.

Although sprint training from a practical point of view is a well known procedure it was thought of interest to further define the protocol applied. Therefore  $V_{O_2}$ , HR and blood lactate concentration were determined at appropriate intervals during one training session (subject H III 100 × 5 s, speed 24 km/h, inclination 11°, rest periods 55 s) using standard procedures employed in our laboratory (see e.g. Christensen *et al.* 1960).

All tests and analyses were carried out before the start of the training and 2 days after the termination of the training period according to the same protocol.

**Functional and anthropometrical tests** The dynamic strength in the legs was assessed by means of Sargent's jump, i.e. vertical jumps from a squatted position, and by Margaria test (Margaria, Aghemo and Rovelli 1966), i.e. running at maximal speed in a staircase with 36 cm step height and 33° inclination. The isometric leg strength was measured as maximal voluntary isometric contraction (MVC) and as endurance time at 50% of MVC (Karlsson and Ollander 1972). Sprint acceleration tests over 25 m were performed using conventional sprint starting positions. The timing in the sprint and in the Margaria test was done with 0.001 s accuracy on a digital timer (TF 2414/1 Markon Instrument Ltd). Individual maximal oxygen uptakes ( $V_{O_2 \max}$ ) were determined on a bicycle ergometer. Expired air was collected in Douglas bags and gas analyses were carried out on a Haldane apparatus (Åstrand and Saltin 1961).

To evaluate gross anthropometrical changes due to the training body weight, height, and thigh circumference of both legs were determined. Thigh girth was measured in a horizontal plane just below the lowest point in the gluteal furrow.

**Biochemical and histochemical analyses** Muscle biopsies were obtained from the lateral portion of the thigh (m. vastus lateralis) (Bergström 1966). The biopsies for the metabolite and enzyme activity analyses were immediately frozen in liquid nitrogen (within 3–4 s) and stored at  $-80^\circ\text{C}$ . The enzyme activities of  $\text{Mg}^{2+}$  stimulated myofibrillar ATPase, CPK, MK and lactate dehydrogenase LDH (EC 1.1.1.7) as well as the concentrations of ATP and creatine phosphate (CP) in the muscle were determined according to the Lowry techniques (Lowry and Passonneau 1972) as modified in our laboratory by Karlsson (1971). The biopsies for the histochemical studies were mounted and frozen in isopentane. Sections of 10  $\mu\text{m}$  were cut and stained for identification of slow twitch (ST) and fast twitch (FT) muscle fibres and estimation of fibre area (for ref. see Gollnick *et al.* 1972).

To assess fibre recruitment pattern during this extreme type of exercise selective glycogen depletion was evaluated by means of PAS staining (Gollnick *et al.* 1974a) of biopsy specimens obtained before and after the training session described above (subject H O).

**Statistical methods** Ordinary statistical methods were applied for calculation of mean values and standard error of the means. Intraindividual differences between values before and after training were tested by the Student's *t* test. In testing hypothesis one-tailed tests have been used (Ferguson 1966, Siegel 1956).

## Results

The results from the single training session (Fig. 1) are in general agreement with what has previously been reported for similar intermittent exercise (Christensen *et al.* 1960, Saltin and Essen 1971).  $V_{O_2}$  during activity averaged 2.8 l/min or approximately 70% of  $V_{O_2 \max}$ .

Subject H.Q. 100 5 sec. [24 km/h IT incl.] 65 sec rest

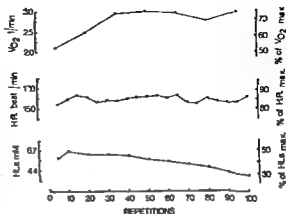


Fig 1 Oxygen uptake heart rate and blood lactate concentration for subject H.Q. performing 100  $\times$  5 s sprint running (rest intervals 55 s) with a speed of 24 km/h and an inclination of 11 presented in absolute figures and in percent of maximal values.

whereas the corresponding value for the rest periods was 2.0 l/min. Blood lactate after every 10th run ranged 4.3–6.7 mM with a mean of 5.6 mM. The relatively high lactates might be related to the fact that the exercise intensity in the present experiment exceeded that applied by e.g. Saltin and Essén (1971).

Both ST and FT fibres showed glycogen depletion after the training session. This indicates that both fibre types were recruited to produce muscle tension during the sprint type exercise performed in the present study.

All subjects demonstrated increases in body weight (mean value 1.4 kg) after the training period, whereas no changes in height were observed (Table I). Simultaneously there was an increase in thigh circumference with on average 1.5 cm (Table I). This indicates that the increase in body weight at least partly was attributable to an augmented leg muscle mass. Increases in MVC Sargent's jump and endurance at 50% MVC were demonstrated for all subjects, whereas 3 subjects improved their performance in the Margaria and the sprint tests (Table II). Consequently the sprint training program improved not only dynamic but also isometric performance. The relative distribution of fibre types was not altered as a result

TABLE I Anthropometrical and histological measurements and muscle phosphagen concentrations before and after 8 weeks of sprint training, and intraindividual differences (d) presented as means and standard error of means (S.E.) and the probability (p) of finding a t value more extreme than the observed under the null hypothesis ( $ns = p > 0.1$ ) ( $n = 4$ ).

	Before	After	d	p
Body weight, kg	66.4 $\pm$ 3.0	67.8 $\pm$ 3.1	1.4 $\pm$ 0.2	< 0.01
Body height, cm	179.0 $\pm$ 3.67	179.1 $\pm$ 3.63	0.1 $\pm$ 0.13	ns
Thigh girth, cm	53.0 $\pm$ 1.60	54.5 $\pm$ 1.11	1.5 $\pm$ 0.31	< 0.05
FT	59 $\pm$ 7.3	56 $\pm$ 7.4	3 $\pm$ 2.1	ns
Fibre area $\mu m^2$ ST	5060 $\pm$ 33.4	5190 $\pm$ 183.8	130 $\pm$ 44.0	ns
FT	5500 $\pm$ 283.2	5740 $\pm$ 316.1	240 $\pm$ 446.4	ns
$V_{O_2}$ max ml $kg^{-1} \times min$	53.7 $\pm$ 2.05	55.4 $\pm$ 2.35	1.7 $\pm$ 1.18	ns
ATP mmol $g^{-1}$	5.1 $\pm$ 0.30	5.4 $\pm$ 0.6	0.3 $\pm$ 0.2	ns
CP mmol $g^{-1}$	19.9 $\pm$ 0.7*	17.5 $\pm$ 0.59	2.4 $\pm$ 1.75	ns

TABLE II Functional tests before and after 8 weeks of sprint training presented as in Table I (n=4)

	Before	After	d	p
MVC kp	165±11.5	184±20.6	19.0±9.21	<0.1
50 MVC sec	47±3.4	56±2.4	9±3.78	<0.05
Sargent's jump cm	47±2.3	51±1.6	4±1.36	<0.05
Margaria test kpm×s <sup>-1</sup>	107.9±3.98	112.5±3.25	4.6±2.32	<0.1
25 m sprint sec	3.78±0.079	3.75±0.063	0.03±0.018	<0.1

of the training (Table I) which is in accordance with other training studies on endurance (Gollnick *et al.* 1973) as well as strength training (Thorstensson and Karlsson 1974). Fibre areas of ST as well as FT fibres on the other hand indicated increases in 3 subjects which might suggest that the leg muscle hypertrophy presented above was related to training induced growth of both ST and FT fibres. Although the muscle concentrations of ATP and CP remained unchanged as compared to before training (Table I) the total amount of phosphagens was increased as a result of the augmented muscle mass. Training effects were demonstrated in the activities of the selected high energy phosphate transferring enzymes (Table III). Increases corresponding to 30, 36 and 20% were shown for Mg<sup>2+</sup> stimulated ATPase, creatine phosphokinase and myokinase respectively.

### Discussion

The major finding in the present study was the qualitative changes in the muscle tissue expressed by the increases in enzyme activity. The significance of these differences were of the same order as for the improvements in muscle performance. In addition it seems reasonable to suggest muscle hypertrophy based on the increased leg muscle circumference. Whether this was related to an increased fibre area cannot be concluded but seems reasonable.

In the present study no attempts were made to separate the protein complexes involved in the contraction machinery as e.g. described by Gergely (1968) and as recently presented by Taylor, Essén and Saltin (1974) for purified myosin. The reason for this was that any similar separation procedure would exclude the possibility of examining the complete protein system of the contraction apparatus of the myofibril. Mg<sup>2+</sup> stimulated ATPase activity as determined in the present study is considered to be a representative measurement of actomyosin ATPase activity in the muscle (Ebashi 1972). The demonstrated increase in the

TABLE III Enzyme activities before and after 8 weeks of sprint training presented as in Table I (n=4)

	Enzyme activity moles 10 <sup>-4</sup> g <sup>-1</sup> min <sup>-1</sup>			
	Before	After	d	p
Mg <sup>2+</sup> ATPase	0.070±0.0046	0.091±0.0051	0.021±0.0088	<0.05
MK	1.86±0.38	2.3±0.231	0.37±0.134	<0.05
CPK	0.99±0.150	1.35±0.131	0.36±0.19	<0.1
LDH	1.56±0.68	1.66±0.8	0.10±0.351	ns

activity of this enzyme would consequently be an acceptable indicator of changes in contractility on the myofibrillar level due to the training. Taylor, Essen and Saltin (1974) presented a relationship between  $\text{Ca}^{2+}$  stimulated myosin ATPase activity and muscle fibre composition which gave no or very little space for the possibility of training effects on myosin ATPase activity according to the authors' conclusion. An explanation to our findings on the basis of changes in the individual proteins involved in the muscle contraction considering the suggested unchanged myosin ATPase activity cannot be given at the present stage of these studies in progress.

In addition to  $\text{Mg}^{2+}$  stimulated ATPase activity, MK and CPK activities have been determined to give as complete a description as presently possible of the enzymes most probably of significance in the immediate turn-over of ATP quanta in relation to sprint type exercise. The rationale for this would be that these enzymes are catalyzing reactions replenishing ATP from ADP (MK) and CP (CPK) respectively. A biological implication of our findings of increased MK and CPK activities with training would then be to enhance ATP resynthesis for extreme muscle activities such as sprint type exercise.

Whether the resynthesis of ATP from ADP and CP is faster than the ATP regeneration from the anaerobic glycolytic pathway is at present uncertain. LDH activity was examined as a potential marker of the anaerobic glycolysis (Gollnick *et al.* 1974 b) but no enzyme activity changes due to the training could be detected (Table III).

As already pointed out, the subjects were in late adolescence and consequently the possibility of growth during the training study has to be considered. No changes in height were, however, present. The observed changes in leg muscle circumference as well as strength parameters seem then to be related to the training procedure rather than growth and/or maturation. To which extent the changes in muscle strength were due to the suggested increases in muscle mass or to the qualitative changes in terms of enzyme activities or both has to be the subject for further investigations.

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## Reduction of the Tone of the Isolated Human Umbilical Artery by Indomethacin, Eicosa-5,8,11,14-Tetraenoic Acid and Polyphlorethin Phosphate

By

KJELL STRANDBERG and TORSTEN TUVEMO

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### Abstract

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STRANDBERG K and T TUVEMO *Reduction of the tone of the isolated human umbilical artery by indomethacin eicosa 5 8 11 14 tetraenoic acid and polyphlorethin phosphate* Acta physiol scand 1975 94 319-326

The effects of 2 prostaglandin synthetase inhibitors indomethacin and eicosa 5 8 11 14 tetraenoic acid (ETA) and of the prostaglandin antagonist polyphlorethin phosphate (PPP) on the tone of the isolated human umbilical artery and on the responses of this preparation to 5 hydroxytryptamine (5 HT) and prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) were investigated. Indomethacin ( $1 \mu\text{g/ml}$ ) ETA ( $5 \mu\text{g/ml}$ ) or PPP ( $40 \mu\text{g/ml}$ ) reduced the tone of human umbilical arteries but had no influence on the responses to 5 HT. In these concentrations ETA and PPP but not indomethacin antagonized the action of  $PGF_{2\alpha}$ . When the concentration of indomethacin or PPP was increased 5 fold both 5 HT and  $PGF_{2\alpha}$  induced contractions were antagonized indicating a non-specific inhibition at these concentration levels. A 10-fold increase in the concentration of ETA had no antagonizing action on 5 HT induced contractions suggesting a more selective inhibition of the  $PGF$  action than displayed by the other compounds. The effects on the tone of the human umbilical artery of the compounds studied might reflect inhibition of prostaglandin biosynthesis and/or antagonism of the action of formed prostaglandins. The findings are compatible with the view that intramural synthesis of prostaglandins contributes to the maintenance of the tone of the isolated human umbilical artery.

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It has been suggested that intramural synthesis of prostaglandins (PGs) maintains the resting tone of gastrointestinal smooth muscle. This hypothesis was based on the findings that PGs are generated in isolated intestinal tissues and that concomitantly with the inhibition by indomethacin of this biosynthesis there is a reduction in tone (Ferreira, Herman and Vane 1972; Eckenfels and Vane 1972).

Tuvemo and Wide (1973) found  $PGF_{2\alpha}$  activity as measured by radioimmunoassay in the bath fluid of the isolated human umbilical artery. Addition of indomethacin inhibited the appearance of PGs and the resting tone of the preparations was reduced. An intramural

synthesis of PGs in the human umbilical artery might have significance for the regulation of the umbilical blood flow as the PGs have been shown to exert both constrictor and dilator actions on this vessel (Altura *et al* 1972 Hillier and Karim 1968 Park Rishor and Dyer 1972)

To further analyse the possibility that PG formation contributes to the maintenance of the tone of the isolated human umbilical artery we have studied the effect on this preparation of 2 chemically unrelated PG synthetase inhibitors indomethacin (Vane 1971) and eicosa 5,8,11,14-tetraynoic acid (ETA) (Abern and Downing 1970) and of polyphloretin phosphate (PPP) a polymer with PG antagonizing action (for references see Eakins and Sanner 1977). By also investigating the effects of the compounds on the contractile responses to  $\text{PGF}_{2\alpha}$  and 5 hydroxytryptamine (5 HT) an evaluation of their specificity was obtained.

### Material and Methods

#### Preparation

Human umbilical cords from full term deliveries were taken immediately after cord clamping. They were transported to the laboratory in Krebs-bicarbonate-glucose solution (KBG) as described by Furchgott (1960). From the fetal part ( $<40$  cm) of the cord spiral artery strips were carefully prepared (Furchgott 1960). They were transferred to 5 ml organ baths and mounted for isotonic recording using a Harvard smooth muscle transducer and a Servogor II recorder. The load was 1 g. The preparations were usually mounted within 10–20 min after delivery. The organ baths contained KBG which was aerated with a gas mixture of 15%  $\text{O}_2$  and 5%  $\text{CO}_2$  in  $\text{N}_2$  giving a  $\text{pO}_2$  value of about 100 mm Hg and a  $\text{pCO}_2$  value of about 40 mm Hg. The temperature was kept at 37°C and the pH at 7.30–7.40. For this study 192 strips from 113 umbilical cords were used.

#### Standardization

Standardization was carried out as follows:

1. After an equilibration period of 60 min in the bath 5 HT (0.2  $\mu\text{g/ml}$ ) was given and 15 min later washed out. Papaverine (100  $\mu\text{g/ml}$ ) was then added to the bath and left for 60 min. Due to the long lasting nature of the complete experiments single doses of 5 HT and papaverine were used for the standardization. The concentrations of the agonists were selected to give submaximal responses rather than supramaximal responses in order to retain reactivity for both constrictor and relaxing effects. The tone level reached after 60 min exposure for papaverine was designated the zero level (0%) and that after 15 min with 5 HT the standard response level (100%). All results presented are expressed in percentages of the standard response.

#### Effects on tone

The effect of indomethacin (Merck Sharp & Dohme) eicosa 5,8,11,14-tetraynoic acid (ETA) (Hoffmann-La Roche Inc.) or polyphloretin phosphate (PPP) (batch 101 k AB Leo Helsingborg Sweden) on the tone of the isolated human umbilical artery was studied as follows:

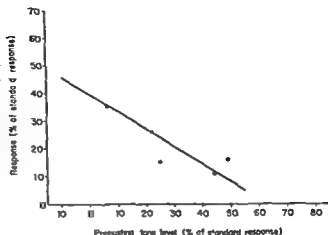
After standardization cumulative dose-response relations for either 5 HT or  $\text{PGF}_{2\alpha}$  were established. After washing the onset of a steady decline or a steady state in tone was awaited. Then indomethacin, ETA or PPP was added to the bath. The effect on tone of the drug was calculated as the difference between the level obtained 20 min after drug addition and the level expected at that time if the steady decline (or steady state) had continued.

#### Specificity

For analysis of a possible inhibiting action of indomethacin, ETA and PPP on the responses of the test preparation to agonists it was primarily decided to compare the dose-response relations for the agonists in the presence and absence of the inhibitors. However this approach was not feasible for the following reasons:

Firstly the magnitude of the contractile responses to the agonists were found to vary significantly with the preexisting tone as illustrated in Fig. 1. Hence it was concluded that dose-response curves could be compared only when they originated from the same level of tone. Secondly preliminary experiments showed

Fig. 1 Relation between the contractile response and the pre-existing tone level. Single doses ( $2 \mu\text{g/ml}$ ) of 5 HT were administered at different tone levels of the preparations. Washing followed on recording of each response. Results from experiments on seven preparations, denoted by different symbols, are presented. For clarification the calculated mean regression line is shown. For each experiment  $r \geq 0.96$   $p < 0.001$ .



that many strips resumed a submaximal tone on washing out the papaverine after the standardization procedure. To compare dose-response curves starting from a high level of tone would not be appropriate since at this level large increases in concentration of an agonist are likely to produce only small increases in response. Thirdly it was discovered (see Results) that addition of an inhibitor to the bath reduced the tone of the test preparation, and more so at a high level. Thus introduction of an inhibitor to the test system might tend to antagonize the smooth muscle contracting action of any added agonist. To overcome these problems it was necessary to start the dose-response curves from a low level of tone and to compensate for the tone reducing effect of the inhibitors under study. For this purpose the control cumulative dose-response curve was produced immediately after the termination of the standardization procedure, i.e. at a low starting level of tone produced by retaining the papaverine in the bath. After the control dose-response relations had been established the strip was washed and an inhibitor was added to the bath. When the tone had decreased to the level at which the control dose-response curve started the second dose-response curve was produced. Thus of the two dose-response curves produced in each experiment, one was constructed in the presence of papaverine and the other in the presence of an inhibitor. The influence of retaining the papaverine ( $100 \mu\text{g/ml}$ ) was estimated in 6 crossover experiments in which  $\text{PGF}_{2\alpha}$  was used as the agonist (Fig. 2). Papaverine produced a parallel shift of the dose response curve to the right ( $p < 0.05$ ).

#### Reproduction

A contraction-relaxation cycle took 1 1/2–2 h. To assess the influence of the time factor on the responses the agonists dose-response relations were established twice with an interval of 1 1/2–2 1/2 h for a total of 9 preparations. The dose-response curves did not differ significantly as tested by analysis of covariance of the differences in response.

#### Statistical analysis

Determination of regression coefficients and analysis of covariance were performed as described by Dixon and Massey (1957).

## Results

### Effects on arterial tone

Indomethacin, ETA and PPP all reduced the tone of the arterial strips. This action is illustrated in Fig. 3. The lowest concentration that consistently produced relaxation was for indomethacin  $8 \mu\text{g/ml}$ , ETA  $5 \mu\text{g/ml}$  and for PPP  $40 \mu\text{g/ml}$ . These concentrations of the

The statistical calculation (analysis of covariance) was based on the differences between the individual responses obtained in the presence and in the absence of papaverine.



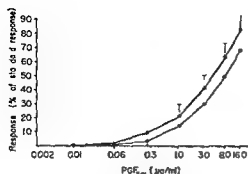


Fig 2

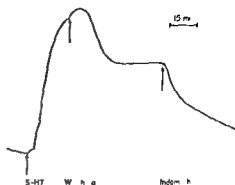


Fig 3

Fig 2 Effect of papaverine on the contractile response to  $\text{PGF}_{2\alpha}$ . In each experiment 2 cumulative dose-response curves to  $\text{PGF}_{2\alpha}$  were determined one with papaverine ( $100 \mu\text{g/ml}$ ) O—O and one without papaverine ★—★ using a cross over design. Means and standard errors of the means for 6 expts are given.

Fig 3 Reducing effect of indomethacin ( $8 \mu\text{g/ml}$ ) on the tone of the arterial strip. The contractile response was produced by adding increasing amounts of 5 HT in total  $3 \mu\text{g/ml}$  to the bath over a period of 18 min starting as indicated by an arrow.

inhibitors produced reduction in the tone by on the average 16% to 36% as calculated for a 20 min period (Table I). This effect seemed to be more marked when the pre-existing tone was high.

#### Effects on 5 HT and $\text{PGF}$ action

The responses to 5 HT were not inhibited by indomethacin  $8 \mu\text{g/ml}$ , ETA  $5 \mu\text{g/ml}$  or PPP  $40 \mu\text{g/ml}$  as compared to the responses produced in the presence of papaverine (Fig 4 a-c). On the contrary the responses were somewhat higher which most likely in part reflected the inhibitory effect of papaverine. In an attempt to exclude the effect of papaverine from the analysis twelve experiments were carried out as follows. The papaverine was washed out after the standardization and the first dose-response curve was constructed. This was followed by washing and indomethacin ( $8 \mu\text{g/ml}$ ) was added as before. Then the second dose-response curve was produced. In only 4 of the 12 expts the level of tone was comparable at the points where the 2 dose-response curves were started. No significant difference between the responses to 5-HT with or without indomethacin in the bath was obtained.

TABLE I Reducing effects of indomethacin ( $n=16$ ), ETA ( $n=14$ ) and PPP ( $n=12$ ) on the tone of the isolated human umbilical artery. The results were calculated as the difference between the tone levels reached after 20 min and the levels expected if no inhibitor had been given. The data for each inhibitor were subdivided into 2 groups of equal size as based on the initial tone of the individual experiments. Means and ranges are presented.

Compound	Tone reducing effect (% of standard response)	
	low initial tone	high initial tone
Indomethacin ( $8 \mu\text{g/ml}$ )	1 (7-46)	34 (17-77)
ETA ( $5 \mu\text{g/ml}$ )	23 (14-33)	36 (18-69)
PPP ( $40 \mu\text{g/ml}$ )	16 (11-6)	7 (12-43)

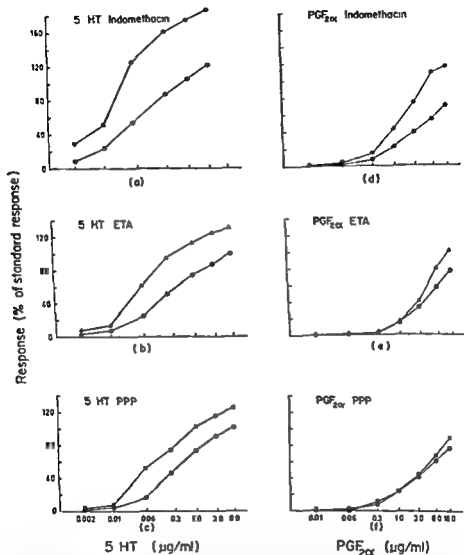


Fig 4 Cumulative dose response curves for 5 HT (a-c) and PGF<sub>2α</sub> (d-f) in the presence of indomethacin (8  $\mu\text{g/ml}$ ) ●—● ETA (5  $\mu\text{g/ml}$ ) ▲—▲ or PPP (40  $\mu\text{g/ml}$ ) ■—■ compared to controls (with papaverine 100  $\mu\text{g/ml}$  present) ○—○ Each curve represents the mean of 5-10 expts

The dose-response curves for PGF<sub>2α</sub> in the presence or absence of inhibitor are presented in Fig 4 d-f. As for 5 HT indomethacin (8  $\mu\text{g/ml}$ ) did not produce any inhibition of the PGF<sub>2α</sub> induced responses. In contrast the dose-response curves for PGF<sub>2α</sub> were almost the same with or without ETA (5  $\mu\text{g/ml}$ ) or PPP (40  $\mu\text{g/ml}$ ). In view of the expected effect of papaverine corresponding to that seen in the experiments with 5 HT the latter results indicated an antagonizing effect of these concentrations of ETA and PPP on the PGF<sub>2α</sub> action. The inhibitory effects of the compounds, now also including indomethacin were

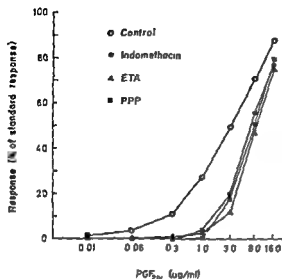


Fig 5 Cumulative dose-response curves for PGF<sub>2α</sub> with indomethacin (40 µg/ml) ETA (50 µg/ml) or PPP (200 µg/ml) compared to control (with papaverine 100 µg/ml present) Each curve represents the mean of 4-5 experiments

clearly demonstrated when the concentrations were increased 5 fold (indomethacin PPP) or 10-fold (ETA) (Fig 5) However at these concentrations indomethacin and PPP but not ETA antagonized the effects of 5 HT as well which indicated a non specific action

### Discussion

Since the discovery by Vane (1971) that certain non steroidal anti inflammatory compounds possess PG synthetase inhibitory activity these compounds have been employed as probes to elucidate the physiological significance of the PGs (*cf* Vane 1973) The mechanism of action of the compounds in this respect is not known in detail but is regarded to be different to that of ETA (Lands *et al* 1973) As yet the PG antagonists available have been of limited value within this field of research They have either suffered from lack of specificity or have been active only in certain tissues and species (*cf* Eakins and Sanner 1972) However some of the PG synthetase inhibitors have effects in addition to that of suppressing the biosynthesis of the PGs Thus high concentrations of *e g* indomethacin and salicylates uncouple oxidative phosphorylation (Whitehouse 1964 Smith and Dawkins 1971) Moreover indomethacin has been reported to exert a membrane stabilizing effect possibly related to an action on Ca<sup>++</sup> fluxes on guinea pig aortic and stomach smooth muscle (Northover 1971) The hazard in using the present PG synthetase inhibitors and PG antagonists for evaluations of the function of the PGs is thus obvious and the results must with necessity be interpreted with caution

The compounds used in this study were selected on the basis that they have different modes of action with respect to PG inhibition All of them (*i e* indomethacin ETA and PPP) were found to reduce the tone of the arterial strips in concentrations which did not antagonize the action of the non PG agonist 5 HT This was taken to indicate that none of the compounds acted via non specific depression of the activity of the smooth muscle cells

In addition ETA and PIP but not indomethacin exhibited an apparent antagonizing effect on the  $\text{PGF}_{2\alpha}$  induced contractions. Similarly Park and Dyer (1973) found an inhibitory effect of PPP (10  $\mu\text{g/ml}$ ) on  $\text{PGE}_2$ -induced contractions of the isolated human umbilical artery but no effect on the responses to 5-HT. When in their experiments the concentration of PPP was raised to 100  $\mu\text{g/ml}$  the responses to 5-HT were also antagonized indicating a low degree of selectivity for PPP. This was confirmed in our experiments. ETA was found more selective than PPP whereas the antagonizing action of indomethacin displayed in higher concentrations seemed to be non-selective.

The finding of a reduced tone of the arterial strips on administration of the lower doses of either indomethacin, ETA or PPP is compatible with the view that an intramural synthesis of prostaglandins controls the level of tone. Indomethacin has previously been shown to abolish the production of  $\text{PGF}_{2\alpha}$  activity in this preparation, as measured by radioimmunoassay (Tuvemo and Wide 1973). The two other compounds might also act via inhibition of the PG synthetase although an antagonism of the action of formed PGs cannot be excluded. Thus ETA has been shown to inhibit PG synthesis in the rabbit heart (Samuelsson and Wennmalm 1971), the cat spleen (Hedqvist, Stjärne and Wennmalm 1971) and to reduce the efflux of urinary  $\text{PGE}_2$  in the rabbit (Larsson and Ånggård 1974).

The mode of action of PPP in the present system is difficult to specify. The compound was originally introduced as a high molecular weight enzyme inhibitor capable of inhibiting several enzymes, e.g. alkaline phosphatase and hyaluronidase (Diczfalussy *et al.* 1953). In addition to its PG antagonizing action, it has recently been found to possess inhibitory activities for both PG-dehydrogenase (Marazzi and Matschinsky 1972; Crutchley and Piper 1973) and PG synthetase (Perklev 1973).

If an intramural synthesis of PGs were of major importance for the maintenance of the tone of the umbilical artery also *in vivo* changes in this production would have impact on the blood flow. Of special interest here is the closure of the artery that takes place at birth. At birth the  $\text{pO}_2$  of umbilical arterial blood is increased. The isolated perfused human umbilical artery has been reported to contract when the  $\text{pO}_2$  is raised (Pamgel 1962). Molecular oxygen is known to be needed for the biosynthesis of PGs (Samuelsson, Granström and Hamberg 1967; Nugteren, Beerthuis and Van Dorp 1967). The possibilities that an increase in the  $\text{pO}_2$  stimulates the biosynthesis of PGs in the umbilical artery and/or promotes the contractile effects of formed PGs are presently under investigation.

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## Effect of Central Depressants on the Acoustic Middle Ear Reflex in Rabbit

### A method for quantitative measurements of drug effect on the CNS

By

E. BORG and A. R. MÖLLER

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#### Abstract

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The respective effects of pentobarbital-sodium, enflurane-sodium, urethane, urethane-chloralose and lidocaine on the function of the acoustic middle ear reflex in the rabbit were studied. The response of the middle ear muscles was measured by recording changes in both ears' acoustic impedance when the reflex was elicited by applying pure tone stimuli (1000 Hz) to the two ears one at a time. In that way both the crossed and the uncrossed reflexes were studied. All the drugs were found to depress the reflex in such a way that a higher sound intensity was required after administration to achieve the same impedance change as before. The effect of the anesthetics was roughly proportional to their known anesthetic power. Lidocaine produced only a slight depression of the reflex. The crossed reflex showed a greater susceptibility to the general anesthetics than did the uncrossed reflex, which suggests a greater complexity of the crossed reflex. Because the method of recording the reflex response does not require any surgery and is equally well applicable in unanesthetized rabbits and in humans, it is suggested as a way of testing the effect of drugs on the central nervous system.

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The activity of the middle ear muscles (stapedius muscle and the tensor tympani muscle) can be measured in man and in the rabbit without any anesthesia or sedation. Measurement of change in the acoustic impedance of the middle ear provides such a possibility and has been found to be both valid and reproducible in this light (Möller 1960, 1961, 1962, Borg 1972 a, b).

A loud sound in one ear elicits a contraction of the middle ear muscles bilaterally though to somewhat lesser extent in the muscles in the contralateral ear (Möller 1961, 1962, Borg 1972 b). In the rabbit the threshold of the ipsilateral reflex is about 70 dB re  $2 \cdot 10^{-4}$  N/m (SPL) for pure tones in the frequency range around 2.0 kHz. Both the stapedius and tensor tympani muscles of this species are activated by sound, the threshold for the tensor tympani being about 10 dB higher than for the stapedius muscle. In man only the stapedius muscle is activated as an acoustic reflex.

The pathway of the acoustic middle ear reflex is in the lower brain stem. The ipsilateral stapedius reflex arc contains 3 neurons. Primary auditory neuron, secondary neuron from ventral cochlear nucleus to the ipsilateral facial nucleus and the motoneuron. The crossed stapedius reflex is activated through one additional relay in the region of the medial superior olivary nucleus. There are in addition parallel multisynaptic connections (Borg 1973).

For further details on the functional properties of the middle ear reflex see e.g. Dallos (1973) and Möller (1972).

Although it has long been known that deep anesthesia abolishes the middle ear reflex, only little quantitative information about the sensitivity of this reflex to anesthetics is available in the literature. In a recent study in man (Borg and Möller 1967) a graded depression of the stapedius reflex excitability was obtained even at small dosages of pentobarbital sodium and ethylalcohol. The reflex recordings in the study of Borg and Möller (1967) were obtained as changes in the acoustic impedance of the ear. Since the same method is applicable to unanesthetized rabbits (Borg and Möller 1968) it was regarded favorable to analyze the effect of various anesthetic agents on the middle ear reflex system in rabbits and hence provide a broader material for purposes of comparison.

In the present study the effect of some central depressants on the acoustic middle ear reflex in the rabbit was investigated. The drugs were general anesthetics and a local anesthetic, general modes of action of which differ. Recordings of the sound-elicited activity of the middle ear muscles were taken immediately before and a certain time after intravenous administration of the drugs.

### Methods

Experiments were performed in a total of 27 rabbits, most of which were used in several experiments with several days in between. The basis for the method used is that the acoustic impedance of the ear is a measure of the mobility of the middle ear system. A contraction of either or both the middle ear muscles changes the mobility of the middle ear system by increasing its stiffness, gradually bringing forth a measurable change in the acoustic impedance. The activity of the middle ear muscles can therefore be recorded by measuring the change in the ear's acoustic impedance, which is largely proportional to the integrated EMG of the middle ear muscles (Borg 1972a).

In the present study the change in acoustic impedance was measured in both ears simultaneously using the same method and set up as have been described earlier (Möller 1961, 1965; Borg and Möller 1968). The two identical measuring devices each included three small hearing aid earphones: one used for producing the measuring tone (800 Hz), the second used as microphone for recording the sound pressure of this tone in the ear canal and the third for delivering the sound stimulus to elicit the reflex (see Fig. 1). The units were connected to the ear by a rubber tube sealed in the ear canal by a dental molding substance.

The acoustic reflex was elicited by pure tones (2 000 Hz) delivered in bursts with a duration of 0.2 or 0.5 s. The intensity was varied in steps of 3 or 4 dB. Both the rise time (10% of maximal amplitude) and the decay time to 10% of maximal amplitude were 10 ms. The acoustic impedance change was measured in both ears simultaneously; the stimuli were presented alternately to the left and right ear at the same time as the intensity was elevated stepwise from below threshold of the reflex to the maximal intensity employed. The intensity was then lowered stepwise to below the threshold of the reflex. In that way a given level of intensity appeared twice.

The level of the stimulus sound at the eardrum depends on the anatomical properties of the individual ears which are likely to vary. Therefore instead of relying on a standard calibration, the stimulus sound pressure was measured in each experiment by using the earphone which usually delivered the 800 Hz measuring tone as a microphone. This microphone, including the narrow tube leading to the ear (see Fig. 1) in turn was calibrated by means of a precision condenser microphone.

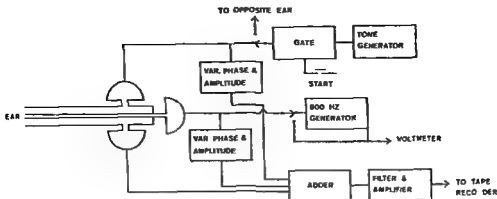


Fig 1 Block scheme of the apparatus for measuring the impedance change

The change in the ear's acoustic impedance caused by a middle ear muscle contraction was measured at a frequency of 800 Hz. The sound intensity of this tone was about 110 dB SPL which is well below the threshold of the acoustic middle ear reflex at 800 Hz. The output of the microphone was balanced out electrically in the absence of the stimulus signal (see Fig 1). When the middle ear muscles contract the ear's acoustic impedance changes and the balance is upset.

The stimulus signal appears in the microphone output circuit with an intensity which is far above the 800 Hz measuring tone. Electrical filters are not sufficient to eliminate an interference of the stimulus tone if the dynamic properties of the reflex are to be reproduced faithfully and therefore the stimulus signal was balanced out electrically in the microphone circuit in a similar way as was the 800 Hz measuring tone (see Fig 1).

The two 800 Hz signals which are a measure of the impedance change in the two ears together with a signal indicating when the stimulus was on were recorded on magnetic tape by a two-track tape recorder and processed at a later occasion.

The amplitude of the impedance change immediately before the end of the stimulus tone was measured and the values in percentage of the maximal obtainable value were plotted as a function of the stimulus level (in dB SPL). The mean of two determinations at each level was used to produce stimulus-response curves.

During the experiments the rabbit was kept in a box open at the top and completely unrestrained. The reflex recordings were performed before and at various times after administration of the drugs.

**Nembutal®** (Abbott (Mebumal sodium)) was administered intravenously dissolved in Ringer solution in concentration of 111 mg/ml. The doses used were 111, 0.30, 40 and 50% of the anesthetic dose (40 mg/kg). **Narcotal®** (Astra, 5.5 mg/ml (Enbomal-sodium)), urethane (100 mg/ml), urethane-chloralose (100 mg/ml and 0.01 g/ml respectively) and **Xylocain®** (Astra, 1 mg/ml (lidocaine)) were administered by the same route. A hyperdermic needle was cut and inserted in a vein in the pinna. A plastic tube connected the cut end of the needle via a 2 way stopcock to two syringes, one with the drug under investigation and one with Ringer solution. The plastic tube was flushed with Ringer solution after each administration.

## Results

### NEMBUTAL

22 rabbits (1.2–2.8 kg body weight) were used in a total number of 44 experimental sessions.

#### Sensitivity of the reflex

The change in acoustic impedance was recorded immediately before and again 5 min after the Nembutal administration. Fig. 2 illustrates a typical experiment in which the impedance change in both ears was recorded simultaneously when a pure tone of various intensities was presented to one ear. Solid lines show ipsilateral and dashed lines contralateral response.



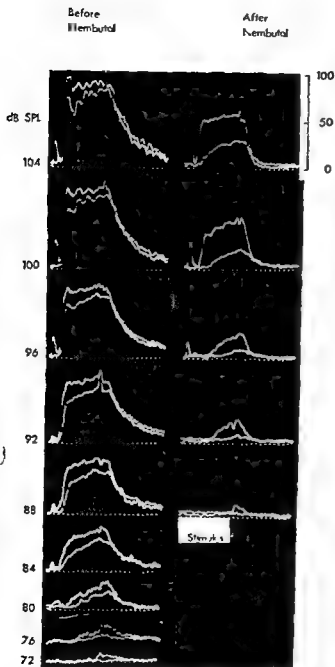


Fig. 2. Change in acoustic impedance at various stimulus intensities as a function of time from before to 5 min after intravenous administration of 16 mg/kg Nembutal. The solid lines represent the impedance change in the ipsilateral ear. The stimulus was a 2000 Hz tone with a duration of 200 ms. The scale in the upper right corner gives the impedance change in percent of the maximal obtainable change.

When the magnetic type recordings were played back the output of the two channels representing the impedance change of the two ears were aligned with respect to amplitude in such a way that the maximal impedance change obtained gave the same deflection for the two ears. The left column shows recordings immediately before administration of Nembutal and the right column recordings 5 min after administration of 16 mg/kg Nembutal.

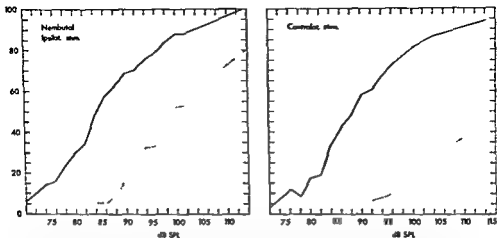


Fig. 3 Impedance change as a function of stimulus sound pressure level before (solid line) and 5 min after intravenous administration of 16 mg/kg pentobarbital. The response was elicited from the ipsilateral ear (left graph) and from the contralateral ear (right graph) with 200 ms long bursts of 2 000 Hz pure tones

The stimulus strength given in sound pressure level in dB SPL, measured near the eardrum, is shown to the left

It is seen in the left graph of Fig. 2 that the magnitude of the response increases with increasing stimulus strength and that the contralateral response to the same stimulus strength is smaller than the ipsilateral. The administration of Nembutal (right graph in Fig. 2) is followed by a decrease of the amplitude of the impedance change for the same stimulus strength and a rise of the threshold. Furthermore the contralateral response is affected more than the ipsilateral response. After Nembutal the time course of the response is changed in such a way that the onset is slower and the offset much faster. The rapid offset probably signifies elimination of slowly conducting parallel pathways.

Fig. 3 shows the stimulus-response-curves of the ipsilateral and contralateral reflex before (solid lines) and after (dashed lines) administration of 16 mg/kg Nembutal. The stimu-

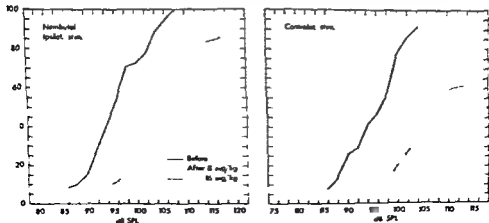
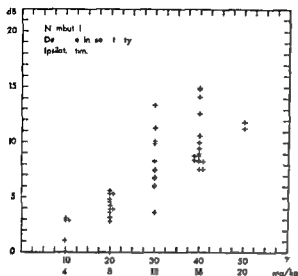
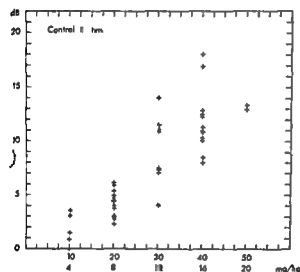


Fig. 4 Stimulus-response-curves before and after 8 mg/kg and 16 mg/kg of pentobarbital.



A



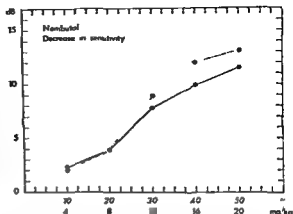
B

Fig. 3. Increase in stimulus intensity necessary to obtain an unchanged response (20% impedance change) of the reflex in ipsilateral (upper graph) and contralateral (lower graph) stimulation at 2000 Hz as a function of a dose of Nembutal.

Intensity-response-curves are shifted to the right which implies that a higher sound intensity is needed in order to obtain the same impedance change as before Nembutal administration. It is further observed that the sensitivity of the crossed reflex is affected more than that of the uncrossed reflex especially at higher sound levels. The sensitivity is reduced in such a way as to get the same impedance change (20% of the maximally obtained) after Nembutal as before an elevation in sound level of about 13 dB being needed for ipsilateral stimulation and 20 dB for contralateral.

Similar results from another experiment are seen in Fig. 4. Two different doses of Nembutal were used here (20% and 40% of that required for surgical anesthesia). A dose of 8

Fig. 6 The mean increase in stimulus intensity necessary to obtain an unchanged response (20% of maximal change) at 2 000 Hz as a function of a dose of Nembutal.



mg/kg reduced the sensitivity of the reflex by about 4 dB (measured at 20  $\Omega$  impedance change). Doubling the dose reduced the sensitivity by about 9 dB. The difference between the sensitivity of the crossed and uncrossed reflex was not as pronounced in this experiment as in that illustrated in Fig. 3.

The effect of Nembutal on the sensitivity of the uncrossed (upper graph) and crossed (lower graph) reflex is shown as a function of dosage in mg/kg in Fig. 5. The change in sensitivity is expressed as the number of dB which the stimulus tone has to be increased after Nembutal in order to elicit the same acoustic impedance change as before (20% of maximal obtainable). The results were obtained in 25 expts. on 11 rabbits. Each point in Fig. 5 represents one ear while Fig. 6 shows the averages of these values. It is evident from these two figures that Nembutal produces a measurable decrease in the sensitivity of the reflex at doses above 4 mg/kg. Furthermore a more pronounced effect on the crossed reflex than on the uncrossed one is seen for doses above 12 mg/kg ( $p < 0.05$  at 16 mg/kg  $p < 0.01$   $t$  test). A potentiating effect of Nembutal on the reflex excitability has not been observed.

#### Time course of the Nembutal effect

The time course of the reflex depression was studied by stimulation with repetitive tone bursts presented at a rate of 1 per 3 s. A constant level of depression was reached some 20

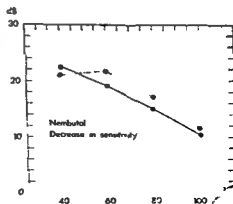


Fig. 7 Increase in stimulus sound pressure of the uncrossed (solid line) and the crossed (dashed line) reflex necessary to obtain an unchanged response (20% of maximal) after intravenous administration of 40 mg/kg Nembutal.

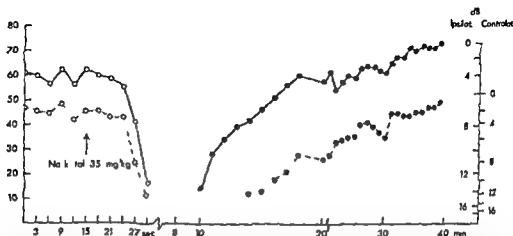


Fig. 8 Impedance change elicited by 200 ms long bursts of a 2 000 Hz tone in percent of maximal obtainable impedance change as a function of time after intravenous administration of 35 mg/kg Narcofol. To the left impedance change and in the right decrease in excitability of the reflex in dB are depicted. The open circles show the response to a tone with an intensity of 86 dB SPL in the left ear of the uncrossed (solid line) and crossed (dashed line) reflex. The filled circles show the response to 92 dB tone.

s after the injection of a subanesthetic dose of Nembutal. The effect lasted at least 5–10 min, i.e. during the routinely performed sensitivity test. The time course of the reflex depression after a single anesthetic dose (40 mg/kg) of Nembutal is shown in Fig. 7. The decrease in excitability was determined from the shift in the stimulus response-curves at 20° of maximal impedance change. No reflex response was present at stimulus intensities below 114 dB SPL until 40 min after the injection of the anesthetic. After 100 min the decrease in sensitivity of the reflex is the same as that seen 5 min after administration of 16–20 mg Nembutal (cf. Fig. 6).

The recovery of the reflex response after a single anesthetic dose of Nembutal was studied in 8 rabbits submitted to middle ear surgery. The loss of sensitivity remaining after about 2 hours was 23 dB for the crossed response and 22 dB for the ipsilateral response.

#### NARCOFOL

The effect of a single dose (35 mg/kg which gives surgical anesthesia) of Narcofol as a function of time was studied in 6 experiments on 3 rabbits. Fig. 8 shows the depression and the restitution of the reflex activity in a typical experiment. The left part of the figure shows the response (impedance change in percent of maximal obtained change) to repetitive stimulation with 2 000 Hz tone bursts with an intensity of 86 dB SPL presented to the left ear. The results shown in the right graph were obtained in a similar way with a sound intensity of 92 dB SPL applied alternately to the left and right. The drug was injected intravenously during 50 s. The beginning of the injection, marked by an arrow, was followed after about 10 s by a depressive action. About 25 s after the beginning of the injection the stimulus strength used did not evoke any measurable response. When the stimulus strength was increased to 92 dB SPL (right part of figure) the ipsilateral reflex reappeared after 10 min and the contralateral after 14 minutes. Complete recovery took place after 30–40 min. A

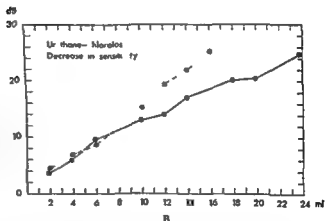
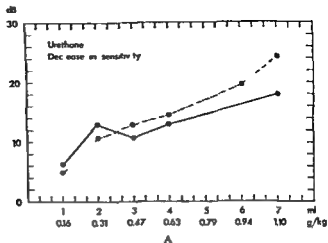


Fig. 9 Increase in stimulus sound pressure necessary to obtain 20% impedance change as a function of a dose of urethane (upper graph) and urethane-chloralose (lower graph) Ipsilateral reflex represented by solid line and contralateral reflex by dashed line Stimulus 2 000 Hz pure tones of 0.0 msec duration One ml urethane-chloralose contains 100 mg urethane and 10 mg chloralose

difference in sensitivity of the ipsilateral and contralateral reflex is seen also after reappearance of the reflex activity. The crossed reflex is about 4 dB less sensitive to stimulation than the uncrossed for as long as about 25 min after the induction of the anesthesia.

#### URETHANE-URETHANE-CHLORALOSE

The effect of these drugs was studied in 1 expt each with cumulative doses. The susceptibility of the reflex was determined from the shift in the stimulus-response-curve at each dose level. Fig. 9 shows the effect of urethane (upper graph) and urethane-chloralose (lower graph) on the uncrossed (solid line) and crossed (dashed line) reflex expressed as the increase in stimulus intensity necessary to obtain a certain impedance change. As is seen the crossed reflex is more susceptible to both drugs especially at higher doses. The addition of chloralose does not seem to decrease the reflex-depressant action of urethane.

#### XYLOCAIN

The effect of intravenously administered Xylocain was studied in 3 expts in 2 rabbits. Fig. 10 shows the stimulus-response-curves of the ipsilateral and contralateral reflex before and

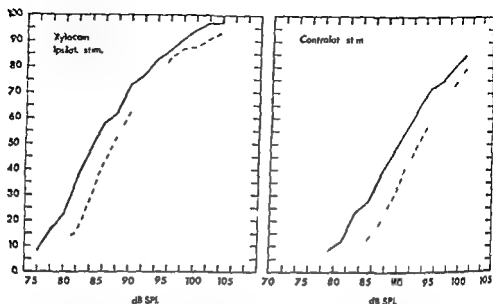


Fig. 10. Stimulus-response-curves before (solid line) and after (dashed line) 10 mg/kg Xylocain i.v.

line) and after (dashed line) 10 mg/kg Xylocain. The curve is shifted to the right equally much for both the crossed and uncrossed reflexes and the excitability is thus decreased about 1 dB.

### Discussion

The results of the present study show that all of the general anesthetics investigated produced a slow and persistent depression of the middle ear reflex. No facilitation was ever observed. The crossed reflex was more sensitive than the uncrossed to all but the smallest doses used. The local anesthetic and antiepileptic Xylocain gave only a slight depression, equal for both the uncrossed and the crossed middle ear reflexes.

Several studies have shown that barbiturates in the doses used in these experiments have no depressant action on striated muscle contractions (Kraatz *et al.* 1953, Borgman *et al.* 1960). Nor are the cochlear potentials changed by anesthetics (see e.g. Lawrence 1960). It is thus possible to ascribe the observed depression of the reflex to an effect on central pathways.

Studies by Arduini and Arduini (1954) and Nakai *et al.* (1965, 1966) have shown that the amplitudes of click evoked potentials both in the brain-stem reticular formation and in the ascending acoustic pathway are depressed by general anesthetics. It is, however, uncertain what is due to action on facilitation-inhibition balance at various locations in the pathway. Multiple unit recordings made at various levels of the auditory system (Hugelin *et al.* 1969) showed that background activity was markedly depressed by anesthetics. The marked depression of the auditory inflow for the middle ear reflex was also observed by Hugelin *et al.*

(1960) and Carmel and Starr (1963) Baust and Berlucchi (1964) found definite changes in the EMG pattern in the tensor tympani reflex after ablation of the acoustic cortex and similar changes were observed by Salomon (1966) after intercollicular decerebration. In the rabbit (Borg 1973) a slow and Nembutal sensitive crossed stapedius reflex response remained after large lesions in the midline of the trapezoid body. This is most likely generated by multisynaptic diffuse connections possibly via the reticular formation.

As pointed out in earlier studies (Møller 1961, Borg and Møller 1967) there are some functional differences between the crossed and uncrossed middle ear reflex, foremost the greater sensitivity of the ipsilateral to acoustic stimuli. The difference in excitability between the ipsilateral and contralateral reflex expresses properties of the parts of the reflex arc that are unique to the contralateral reflex (Møller 1961). The difference between the two reflex arcs is mainly the presence of a direct connection from the ventral cochlear nucleus to the ipsilateral 7th motor nucleus and the absence of any such connection with regard to the contralateral facial motor nucleus. The greater sensitivity of the contralateral reflex to general anesthetics is shown here and by Borg and Møller (1967) answers to the expectations to which these reflex arc differences give rise.

The difference in drug sensitivity between the crossed and uncrossed reflexes derives from the properties unique to the crossed reflex. From Fig. 6 it is seen that at a dose of 16 mg/kg the increase in stimulus intensity necessary to obtain an undiminished impedance change is 4 dB greater for the contralateral reflex than for the ipsilateral.

Intravenously administered Xylocain in doses several times those sufficient to block epileptic seizures had only very slight depressive action on the middle ear reflex. This is in accordance with its demonstrated low effect on other polysynaptic reflexes (Blom 1963).

As seen from Fig. 6 and 9 fifty per cent of the dose for surgical anesthesia of Nembutal urethane and urethane-chloralose gives almost equal depression of the contralateral reflex while Xylocain has in principle no effect on the middle ear reflex or on consciousness. The sensitivity of the middle ear reflex to the drugs studied thus seems to be related to their effect on consciousness, i.e. probably due to an effect on the reticular formation. By comparison the middle ear reflex in humans (Borg and Møller 1967) is much more sensitive to Nembutal than that in the rabbit, taking body weight into account. This finding agrees with the proportions generally assumed, i.e. about 5 times smaller anesthetic dose for human than for rabbits.

It is suggested that measurement of the middle ear muscle reflex excitability can be useful for the determination of drug effects. Both the degree and the time course of the effects can be studied conveniently.

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The occurrence of 2 intermingled sets of capillaries running in parallel and with very little interstitial tissue inbetween them makes the *rete* especially suited for studies of capillary permeability. Such investigations have been performed (Stray Pedersen and Steen 1975, Stray Pedersen 1975). In order to analyze and to understand the data obtained in such studies one needs however structural information on the capillaries under study. It is generally assumed that the permeability characteristics of different types of capillary walls depend on their specific structure which may vary from species to species and especially from one organ to another within the same species (Bennett *et al.* 1959). For the computation of permeability and diffusional constants in the *rete* one will thus need exact informations about the actual size of important structural parameters such as the surface and the thickness of the retial capillary barrier. Ultrastructural studies of the retial vessels have been performed by Dorn (1961) and by Fawcett (1963) in organs fixated by immersion and recently by Bendayan *et al.* (1974) in organs fixated by perfusion. Neither of these studies give however sufficient quantitative information for a satisfactory functional analysis.

In the present study qualitative and quantitative morphological studies have been carried out. To this end light microscopical as well as electron microscopical examinations have been performed. The ultrastructural studies were predominantly carried out in preparations which were fixated through perfusion since this method appears to give a more sudden arrest of the tissue situation than immersion fixation.

The ionic composition of the endothelial cells were evaluated by measuring the concentrations of sodium, potassium, calcium and magnesium in retial tissue and in eel erythrocytes.

### Materials and Methods

**Gross vascular anatomy.** As seen in Fig. 1 the swimbladder of the eel has two *retia* which get their blood supply from a branch of the swimbladder artery, the *pre rete* artery. At the distal pole of the *rete* the arterial capillaries reunite into two or more *post rete* arteries which again divide and give origin to the capillary network of the secretory bladder. The venous blood is presented to the *rete* at its distal pole in the *pre rete* veins which split into numerous venous capillaries running parallel to the arterial ones but in the opposite direction. At the proximal pole of the *rete* the venous capillaries reunite into a single *post rete* vein. Thus the blood to and from the secretory bladder has to pass through a complex vasculature consisting of 3 successive capillary systems or portal systems of which the first and the last ones are situated *in situ*.

*Retia* from eels of different sizes were employed in this investigation. The animals which almost exclusively represented silver eels were completely adapted to fresh water. The eel was either made unconscious by a blow on its neck or else anaesthetized by submersion in a 0.05% solution of MS-222 for 3 min. The eel was fastened in an eel holder (Steen 1963a) and the gills irrigated with tapwater. The abdomen was then opened and the *pre rete* artery and the *post rete* vein cannulated. The vessels distal to the *rete* were cut and the *rete* perfused through the two cannulated vessels.

**Light microscopical studies.** The *retia* were dissected out, fixated in 10% formaline and stained with hematoxylin-eosine (H-E). Cross-sections as well as longitudinal sections of the *retia* were examined.

The quantitative measurements were performed on cross sectioned *retia* fixated in 10% formaline for 4-72 h (section thickness 10  $\mu$ m, stained with H-E). Since it was easier to distinguish between arterial and venous capillaries when the blood cells had been washed out, a short lasting perfusion with plasma or with Ringer solutions containing papaverine in order to paralyze the vasculature (Stray Pedersen 1970) was performed prior to the fixation. The shrinkage due to the fixation was evaluated by comparing the circumferences of the *retia in situ* with those of the corresponding preparations after fixation. The latter type of circumference was measured on the 10  $\mu$ m thick cross-sections which were magnified by a slide projector. Measurements on 10 *retia* of very different sizes showed that corrections of about 10% and 20% were necessary for the length and the area values respectively in the fixed preparations.

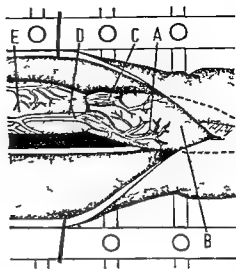


Fig 1 A drawing of the swimbladder of an eel intestines and gonads removed. A=Post rete artery and pre rete vein B=secretory bladder C=rete D=pre rete artery and post rete vein E=reabsorbent bladder

The total cross sectional areas of the *rete* were determined very accurately by putting the microscopical sections into a slide projector and then drawing the outlines of the depicted preparations on mm paper. The actual magnification was determined by means of a micrometer arrangement in the projector.

The number of arterial and venous capillaries was counted through a microscope equipped with an ocular grid. The countings were performed in at least 20 small squares with a side length of 0.0075 cm, each square containing on an average 25 capillaries. In one preparation (preparation E in Table I) the number of capillaries as well as the cross sectional area was also estimated on microphotographs. The countings were performed in superficial as well as in central parts of the *rete*.

From the values obtained for the total number of capillaries ( $N_T$ ) and the cross-sectional area ( $A_C$ ) the mean capillary radius ( $r$ ) in the *rete* could be computed according to the formula

$$r = \sqrt{\frac{2/3 A_C}{\pi N_T}} \quad (1)$$

assuming the area constituted by tissue to be 1/3 of the total cross-sectional area (Krogh 1979). In two preparations the diameters of the capillaries were also measured directly with an ocular micrometer.

The total surface of each capillary system ( $A$ ) in the *rete* was calculated according to the formula

$$A = \frac{1}{2} N_T \cdot 2 \pi r L \quad (2)$$

in which  $L$  represents the capillary length. Assuming  $N_A \approx N_V$ .

**Electron microscopic studies** were performed predominantly on perfusion fixed but also on immersion fixed cross sectioned *rete*. Prior to the fixation the *rete* was perfused with a Ringer solution containing 3% Dextran (Dextran T 500 Pharmacia). The fixation was performed by perfusion of a solution containing 1% glutaraldehyde in 0.08 M phosphate buffer (pH 7.4) for 15–20 min. The *rete* developed a uniform yellow tinge after few min. Samples of the reticular tissue were then immersed for 2 h in ice-cold 3% glutaraldehyde in 0.08 M phosphate buffer, then cut into small blocks of about 1 mm and washed overnight in ice-cold Tyrode buffer. The blocks were post fixed for 1 h and 30 min in ice-cold 1%  $OsO_4$  in Tyrode buffer (pH 7.4), washed briefly in Tyrode buffer, dehydrated in graded alcohols and embedded in Epon 812. Semithin sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were cut with a LKB-Ultratome ultramicrotome, stained with uranyl acetate and lead citrate (Reynolds 1963) and examined in a Siemens Elmiskop I electron microscope.

**Measurements of electrolytes in reticular tissue and *rete***. The *rete* was perfused with plasma or Ringer solutions in order to wash out the blood and in some experiments inflated with air. The capillary distribution of the organ was dissected out, cut into slices each weighing 10–20 mg, which were carefully

TABLE I Cross-sectional areas, number radii and surface areas of capillaries in 5 different retia

Preparation	Cross-sectional area of the rete ( $A_C$ ) (mm <sup>2</sup> )	Number of arterial capillaries ( $N_A$ ) $\pm$ S.D.	Number of venous capillaries ( $N_V$ ) $\pm$ S.D.	Total number of capillaries ( $N_T$ )	Calculated mean capillary radius ( $r$ ) ( $\mu$ ) $r = \sqrt{A_C / 3.14 N_T}$	Measured mean capillary radius ( $R$ ) ( $\mu$ )	Calculated capillary surface area ( $A = 2 \pi r l$ ) (cm <sup>2</sup> )
A	20.56			182 500	4.89		112
B	5.25	33 600 $\pm 4 800$	31 500 $\pm 3 700$	55 100	4.50		31
C	3.88	22 100 $\pm 3 500$	15 200 $\pm 1 500$	37 300	4.70	5.33	20
D	10.70	51 300 $\pm 4 200$	38 000 $\pm 3 100$	89 300	5.05	4.82	57
E	9.15	55 400 $\pm 6 300$	34 400 $\pm 5 400$	89 800	4.65		52
Krogh's rete (Krogh 1929)	8.0	111 000	44 000	102 000	4.08	4.2	52

squeezed on filter paper in order to remove as much intravascular fluid as possible. The tissue slices were dissolved into 200  $\mu$ l of hot concentrated sulphuric acid. 200  $\mu$ l of H<sub>2</sub>O was added and the concentrations of the various ions were measured in this solution. The fact that the various ion concentrations in these solutions were found to be identical with the concentrations measured in the supernatants obtained after centrifugation of this solution indicated that the tissue had become completely disintegrated during the treatment with sulphuric acid. The concentrations of potassium and sodium were determined by flame photometry using an Eppendorf flame photometer. The concentrations of calcium and magnesium were measured with a Unicam atomic absorption photometer. Blank analyses with the solution of concentrated sulphuric acid and water were performed in order to correct for the contamination of sodium from the glass.

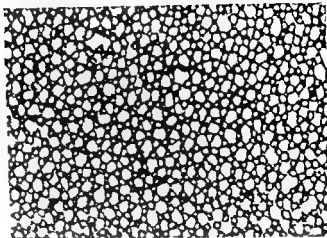
In order to compare the values obtained from retial tissue with those from other homologous cells, similar analyses were performed on eel erythrocytes. The red cell suspension was obtained by centrifuga-

TABLE II The ionic composition of the retial tissue and of eel erythrocytes as compared to that of human erythrocytes

	Eel rete mM/l	Eel erythrocytes mM/l	Human erythrocytes mM/l
K <sup>+</sup>	61 $\pm$ 13 (S.D.) n = 4	111 n = 2	136 [1]
Na <sup>+</sup>	73 $\pm$ 25 (S.D.) n = 4	43 n = 4	19 [1]
Ca <sup>+</sup>	4 n = 2	5 n = 1	0.04 [2]
Mg <sup>++</sup>	9 n = 2	12 n = 1	3.3 [2]

References [1] Bernstein (1954) [2] Harrison and Long (1968)

Fig. 2. Micrograph of a cross sectioned *rete* ( $\times 430$ ) which has been perfused with horse plasma prior to the fixation. The arterial and venous capillaries may easily be identified: the arterial vessels having smaller lumens and thicker walls. The venous capillaries, however, are considerably distended in this preparation as a result of the conditions prevailing during the perfusion.



tion of eel blood at 1000 g for 20 min. The values were corrected for the inevitable contamination of the cell sediment with plasma (4%).

The accuracy of getting ionic composition from biological specimens heated with sulphuric acid was tested by carrying out the same procedure on plasma. The values obtained with this method were seen to be accurate to within  $\pm 10\%$  when compared to the values obtained by direct ionic measurements in plasma. The reliability of the method for measurement of intracellular ion concentrations was evaluated by carrying out similar analyses of the ionic content in human erythrocytes. The values obtained were in the ranges of 144–169 meq/l for  $K^+$  and 12–18 meq/l for  $Na^+$  and thus not essentially different from the values reported by other investigators (Table II).

## Results

### Light microscopical studies

On cross-sectioned preparations the very regular arrangement of the retial capillaries could be recognized (Fig. 2). The arterial and venous capillaries could easily be distinguished in the preparations in which the red cells had been washed out: the arterial capillaries having smaller lumina and thicker walls. An arterial capillary was surrounded by 3 to 7 (in most cases 4) venous ones and *vice versa*. The capillaries located close to the surface did often have a somewhat smaller calibre than the central capillaries. However, the opposite phenomenon was also observed in some preparations. Structures resembling arterio-venous anastomoses were never observed. Nor were discontinuities in the capillary walls ever seen.

On preparations sectioned longitudinally the capillaries were seen to be straight or slightly curved. At both ends of the capillaries numerous melanophores could be detected.

**Quantitative estimations.** Macroscopically the capillary division of the *rete* was distinctly marked by a narrow pigmented zone at both poles due to the presence of numerous melanophores in these areas. The capillary length could therefore be measured directly. In 10 *retia* of very different sizes an almost constant length of 4 mm was obtained.

Measurements of the cross-sectional area and of the number of capillaries were performed on 5 *retia* of very different sizes. In one preparation (preparation A in Table I) it was impossible to distinguish between the arterial and venous capillaries and hence only the total

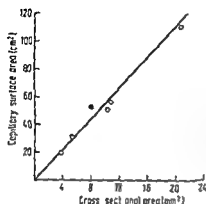


Fig 3 The relationship between the measured values for cross sectional area and the calculated values for total capillary surface area in 5 *retia* (see Table I) (The black circle represents values given by Krogh for the *rete* examined by him (Krogh 1929))

number of capillaries could be estimated. In the remaining four *retia* the arterial and venous capillaries were counted separately. The number of capillaries arrived at as well as the mean capillary radii and surface areas calculated according to Eqs 1 and 2 are listed in Table I and compared to the values obtained by Krogh (1929). Fig 3 shows the relationship between the capillary surface area and the corresponding cross sectional area in these *retia*.

In two preparations (preparations C and D in Table I) the capillary diameters were measured directly ( $n = 100$ ). The diameters were found to be as follows (mean values  $\pm$  S.D.)  $d_A = 8.64 \mu\text{m} \pm 1.43$  and  $d_V = 13.62 \mu\text{m} \pm 1.87$  in preparation C and  $d_A = 8.70 \mu\text{m} \pm 1.25$  and  $d_V = 10.90 \mu\text{m} \pm 2.08$  in preparation D giving mean capillary radii of  $5.33 \mu\text{m}$  and  $4.82 \mu\text{m}$  respectively (calculated according to the formula  $r = (N_A r_A + N_V r_V) / N_T$ ).

total capillary surface in the arterial and venous system in these preparations would be  $A_A = 24 \text{ cm}^2$  and  $A_V = 26 \text{ cm}^2$  in preparation C and  $A_A = 56 \text{ cm}^2$  and  $A_V = 52 \text{ cm}^2$  in preparation D. As seen from Table I these values are not essentially different from those computed from the values of the capillary number and the cross sectional area assuming the retial tissue to represent 1/3 of the cross sectional area (Eqs 1 and 2).

#### Ultrastructural studies

Even at low magnification the arterial and venous capillaries were easily identified (Fig. 4). On cross-sections the arterial capillaries were approximately circular in outline and had rather thick walls. The venous capillaries were more irregular in outline and had considerably thinner walls. In both types of capillaries the main elements of the general structure of capillary walls could be recognized (Fig. 4). The endothelium and the basement membrane were surrounded by a layer of interstitial tissue (adventitia). In each complete cross sectional cut of endothelium parts of some 4-7 endothelial cells could be recognized. In the spaces between the cells the junctions could be observed as darker regions. The endothelial cells contained numerous vesicles and vacuoles thus giving the cytoplasm a spongy appearance. Each capillary was surrounded by a distinct basement membrane whereas the adventitia appeared as a less well defined layer especially in those regions in which the arterial and venous capillaries were in close contact with each other. By far the greatest

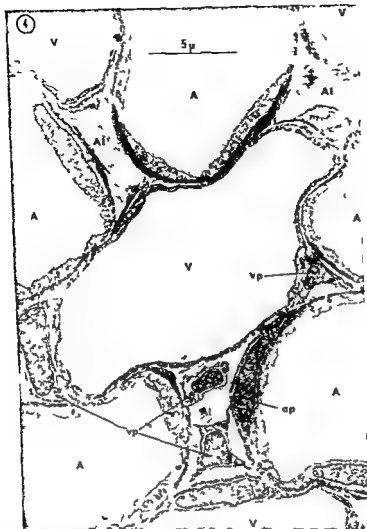


Fig 4 Electron micrograph of a cross sectioned perfused eel rete mirabile showing a venous capillary (V) in center surrounded by 5 arterial capillaries (A). Between the fragments of the endothelial cells the intercellular junctions may be recognized as darker regions. In the angular interstice (AI) at the bottom of the picture a venous pericyte (vp) is seen to be located freely in the interstice whereas the arterial pericyte (ap) appears to be embedded in the basement membrane of the arterial capillary.

fraction of interstitial tissue was confined to the angular interstices which also contained the perikarya of the pericytes (adventitial cells).

Since the endothelia of the arterial and venous capillaries were essentially different in structure they should be described separately.

**Arterial endothelium** The endothelium had its greatest thickness in the nuclear regions (about  $1 \mu\text{m}$ ) and was more or less attenuated towards the periphery the average thickness being about  $0.3 \mu\text{m}$ . At the levels of the intercellular clefts the height of the endo-



was often seen to be markedly diminished in such a fashion as to make the appearance of cavities in these regions

The plasmalemma had a thickness of about 100 Å and did not seem to differ in structure from the usual unit membrane. The structure was uniform along the entire perimeter of the cell and was not affected by local differentiations such as plasmalemmal invaginations or cell protrusions

The cytoplasm contained the usual complement of cell organs. The Golgi complex, the endoplasmatic reticulum, the ribosomes and the mitochondria were generally concentrated in the nuclear region. The ribosomes were predominantly confined to the endoplasmatic reticulum, free ribosomes were rather infrequently encountered. The mitochondria were rare but of prominent sizes

The cytoplasm was crowded with pinocytotic (plasmalemmal) vesicles (*e.g.* Fig. 8-11). The population of the vesicles was greatest in the attenuated parts of the endothelium. The vesicles were circular or ovoid in outline with diameters between 200 and 800 Å and were bounded by a membrane similar to the plasmalemma. They were especially numerous along the two borders of the cell in the immediate vicinity of the cell membrane thus indicating that they represent plasmalemmal invaginations. About 1/3 of the vesicles were located freely in the cytoplasm. Many vesicles were seen to open onto the luminal or sub-endothelial space (Fig. 9) or onto the intercellular clefts (Fig. 6) either directly or through a narrow neck. Some vesicles were separated from these spaces by a diaphragm which was continuous with the cell membrane but considerably thinner than this (about 50 Å) (Fig. 9) others were attached to the surface by strands of plasmalemma. The vesicles contained various amounts of fine granulated material or appeared to be empty. No systematic variations of the vesicles, especially with respect to their sizes and content could be observed along the luminal-abluminal axis. In some places chains of 2-4 intercommunicating vesicles were observed, some of which were seen to open at the cell surface. However a continuous chain of intercommunicating vesicles from the luminal or the intercellular space to the sub-endothelial space was never observed.

The cytoplasm also contained vacuoles of considerably greater sizes (*e.g.* Fig. 4 and 5). These vacuoles were more or less elongated with a width between 0.1-0.3 µm, and a length between 0.1-1.5 µm. Similar to the pinocytotic vesicles they were most frequently located in the peripheral parts of the cell. The content of these large vacuoles appeared to be similar to that observed in the lumen (Fig. 14).

The nuclei of the endothelial cells were seen to be indented and often bent to follow the curvature of the vessels (Fig. 4). The nuclear plasma had a fibrillar or granular structure and contained irregular masses of chromatin.

At the levels of the intercellular clefts the two adjacent endothelial cells would show different degrees of overlapping. The length of the clefts could therefore vary considerably. Some had the appearance of short, straight running channels with a length of about 500 Å (Fig. 5 and 9) whereas other clefts appeared as tortuous slits (Fig. 6) having a length of several µ. The diameter of the clefts varied between 100-250 Å. The clefts appeared to have a random distribution, i.e. they were located just as frequently in the endothelium facing the angular interstices as in other regions of the wall. In some places, however a juxtaposition

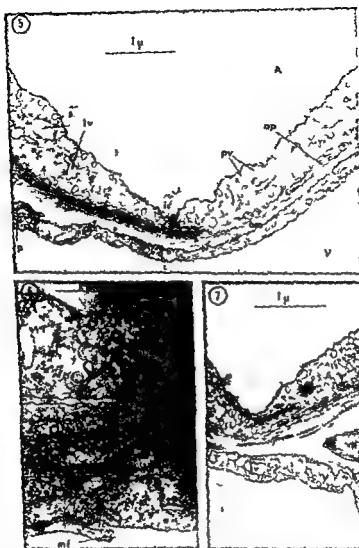


Fig. 5. Electron micrograph of a cross-sectioned perfusion fixated *rete* showing the arterio-venous barrier at a site of minimal barrier. The content of pinocytotic vesicles (pv) is seen to be significantly higher in the arterial than in the venous endothelium. In both endothelia large vacuoles (lv) are observed. In the luminal half of the intercellular cleft (ic) of the arterial capillary a desmosome is recognized.

Fig. 6. Electron micrograph of a cross-sectioned perfusion fixated *rete* showing an arterial and a venous intercellular cleft (ic) in juxtaposition. In the arterial cleft, which appear to have a patent gap junctional structures seem to be absent. A narrowing of the space between the cell membranes may however be observed at a distance of 0.5  $\mu$ m from the luminal surface. Pinocytotic vesicles partly intercommunicating open onto the cleft. In the region close to the venous intercellular cleft a marginal fold (mf) is observed.

Fig. 7. Electron micrograph of a cross-sectioned perfusion fixated *rete* showing an arterial intercellular cleft (ic) with a more centrally located desmosome. At both ends of the desmosome narrowings of the intercellular space are recognized. In these regions (not so distinct in this picture) an increased accumulation of dense material partly obliterating the intercellular space. These structures most probably represent special attachment devices (see text).

of an arterial and a venous cleft as shown in Fig. 6 and 10 was observed. In certain regions of many clefts usually in their luminal thirds elements of denser junctional structures with the appearance of desmosomes could be observed (Fig. 5 and 7). Usually the junctional spaces seemed to contain masses of material with light density. In all the clefts including those where no desmosomes could be seen one could observe a narrow region extending for 100–300 Å. The average diameter of the junctional gaps in these regions was found to be about 110 Å as measured on 20 junctions.

*Venous endothelium.* The endothelium varied considerably in thickness but it was generally found to be thinner than in the arterial capillaries (about 0.15 µm).

In some places (Fig. 9, 12 and 13) smaller regions of discontinuities of the cell wall could be observed. These regions the so-called fenestrae appeared to be similar to those described in some mammalian capillaries (Rhodin 1962, Luft 1965, Maul 1971). The diameters of the fenestrae were between 200–800 Å. Each fenestra was provided with a diaphragm (of about 50 Å in thickness?) which was continuous with the plasmalemma (Fig. 9). A central knob in the diaphragm could not be detected. The fenestrae were most frequently encountered in the attenuated parts of the endothelium especially in those regions in which the total arterio-venous barrier was very thin. Fenestration of the venous capillary walls facing the angular interstices was in fact very uncommon. The number of fenestrae in the venous capillary walls seemed to be much smaller than what has been observed in mammalian visceral capillaries (Friederici 1968, Clementi and Palade 1969). On many of the present venous capillary profiles fenestrae were completely absent. When observed however they were usually arranged in clusters of 3–5.

The cytoplasm and the nucleus of venous capillaries had a less dense structure than in arterial capillaries but the same structural elements were encountered in both types of capillaries. The concentration of pinocytotic vesicles was significantly smaller in venous capillaries though. At the luminal surface which appeared to be more irregular than in the arterial endothelium clusters of microvillous processes could be observed. In many instances as shown in Fig. 6, 12 and 14 such cytoplasmic extrusions could be recognized near the intercellular clefts (marginal folds) and in the close vicinity of one or more giant vacuoles.

The intercellular clefts (Fig. 6, 9 and 11) seemed to be similar to those in the arterial capillaries. In the venous capillaries they appeared to be more straight running and somewhat wider though having diameters between 100 and 300 Å. In their narrow regions the diameters were about 120 Å.

The basement membrane had a thickness of 100–150 mµ and contained numerous microfibrils. On the majority of the micrographs (not so distinct on those shown in the figures) a three layered structure of the membrane such as described by Dorn (1961) could be more or less recognized. In most places the arterial and venous basement membranes appeared as separate distinct structures. However in those regions in which the arterial and venous capillary walls were in intimate contact the basement membranes were seen to be more or less fused. In some instances it was difficult to distinguish between the basement membrane proper and the underlying interstitial tissue in that both elements made up one more or less continuous layer (Fig. 12, 14). Note that the black spots observed in these figures represent accumulations of horse radish peroxidase molecules. It is possible that this layer

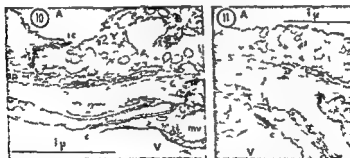
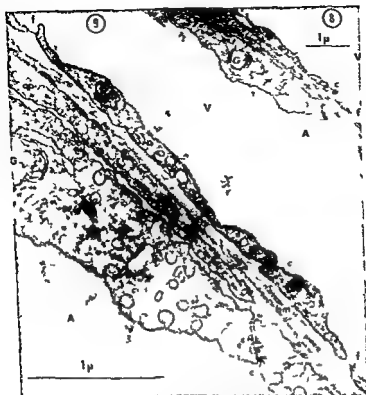


Fig. 8 Electron micrograph of a cross-sectioned perfused rete showing the arterio-venous barrier in a region in which the venous capillary wall shows fenestrations. The venous endothelium is thin and provided with 2, perhaps 3 fenestrations (f) situated close to the intercellular cleft. In the arterial capillary a Golgi apparatus (G) is observed.

Fig. 9 Same as in Fig. 8 with a higher magnification showing details in the structure of the fenestrations (f). The diaphragms of the fenestrations are significantly thinner than the plasmalemma (about 50 Å). The intercellular cleft (c) is very short (about 500 Å) and to contain denser material.

Fig. 10 Electron micrograph of a cross-sectioned perfused rete showing a venous intercellular cleft (ic) having a continuous space. A marginal vacuole (mv) is located in the immediate vicinity of the cleft.

Fig. 11 Electron micrograph of a cross-sectioned perfused rete showing a venous intercellular cleft (ic) with desmosomes.

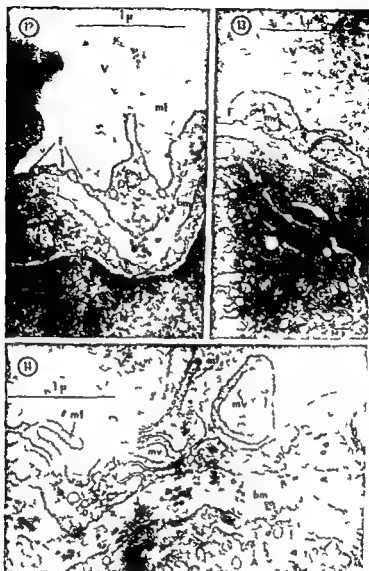


Fig. 1-14 Electron micrograph of cross-sectioned immersion fixed *rat* showing the possible mechanism behind the formation of vacuoles in the venous endothelial cells. A marginal fold (mf) may fuse either with the cell body or with another fold and thus impound droplets of luminal fluid forming a marginal vacuole which then may move towards the central parts of the cell as clear vacuoles. In Fig. 12 and 13 the endothelium is provided with fenestrae (f). The intercellular clefts (ic) in Fig. 13 and 14 appear to have patent gaps. The structure of the basement membrane (bm) is very homogenous in all these figures.

The sections shown in Fig. 1-14 represent a *rat* which had been perfused with plasma containing 100 mg % of horse-radish peroxidase (Type VI). The numerous black spots in the cytoplasm and especially in the basement membrane most probably represent focal concentrations of the peroxidase molecules.

actually consisted of many layers of basement membranes containing no or very little of interstitial tissue but this could not be ascertained in the present study. At the edge of a pseudopodium of a pericyte the basement membrane was seen to split into two leaflets



Fig 15 Electron micrograph of a cross-sectioned perfusion fixed rete showing some structural elements of an angular interstice (AI): Numerous collagenous and elastic fibrils, a bundle of non myelinated nerve fibres (nf) and perikarya of venous (vp) and arterial (ap) pericytes. The arterial pericyte (and its nucleus) has a crescent shape similar to that of the endothelial cells.

which then surrounded the cell. Between the endothelium and the pseudopodium of a pericyte the basement membrane was rather thin and often discontinuous, thus allowing an intimate contact between the endothelial cells and the pericytes.

The adventitial tissue was predominantly confined to the angular interstices. In these regions numerous collagenous and elastic fibres and some non myelinated nerve fibres were observed (Fig. 15). The perikarya of the pericytes were also located in the angular interstices.

(Fig. 4 and 5) These perikarya were seen to make an elaborate system of processes or pseudopodia disposed perpendicular to the long axis of the vessel. The arterial and venous pericytes had large dark nuclei. The cytoplasm contained numerous dense particles (glycogen?) some pinocytotic vesicles and also large vacuoles.

The arterial and venous pericytes could easily be identified (Fig. 4). The arterial pericytes were seen to be in intimate contact with the capillary wall being surrounded by the arterial basement membrane along its entire circumference. The shape of these cells (and also of their nuclei) was therefore similar to that of the endothelial cells. The perikarya of the venous pericytes were seen to have much more irregular outlines and more rounded nuclei. Only their pseudopodia were seen to be surrounded by the (venous) basement membranes, the perikarya being located freely in the angular interstices.

*Measurements of the concentrations of ions in retial tissue.* The concentrations of  $K^+$  and  $Na^+$  were measured in 24 retia of different sizes. In 2 retia measurements of the concentrations of  $Ca^{++}$  and  $Mg^{++}$  were also included. Similar analyses of these four ions were performed on two different samples of cell sediments from eel blood. It should be emphasized however that with our methods the total concentrations of these substances (ionized and non ionized) were measured. This would be of no significance to the values obtained for  $K^+$  and  $Na^+$ . In the case of the bivalent ions we would have to consider that a significant but small fraction would exist as non ionized molecules within the cells. The results obtained are presented in Table II in which the corresponding concentrations in human erythrocytes are quoted.

### Discussion

The present structural studies demonstrate how the *rete mirabile* is optimally constructed for effective capillary exchange. The 2 types of capillaries are arranged so that a maximal surface area is available for the exchange between the arterial and venous system. The total capillary surface area in each capillary system is practically identical as was also reported by Krogh (1929). A *rete* of average size having a cross sectional area of 5 mm<sup>2</sup> and a volume of 21 mm<sup>3</sup> has some 34 000 arterial and 22 000 venous capillaries with a total surface area in each capillary system of about 30 cm<sup>2</sup> (Table I).

The capillaries have an almost uniform length of 4 mm. The linear relationship between the cross-sectional areas of the *retia* and the corresponding calculated capillary surface areas (Fig. 3) indicated that the capillaries have constant calibres. From the values quoted in Table I the mean (inner) diameter of the vessels would be in the range of 9–10  $\mu$ m, the venous capillaries being wider than the arterial ones by a factor of 1.35–1.6. In this respect they are similar to other capillaries investigated in lower vertebrates but wider than mammalian capillaries.

The *rete* contains very little of interstitial tissue. As has been presented in Eq. 1 and as might be accepted from inspection of the micrographs of cross sectioned *retia* the capillary lumina represent about 2/3 and the tissue elements (capillary walls + interstitial tissue) about 1/3 of the cross-sectional area. The correspondence between the radii calculated (according to Eq. 1) and those measured (Table I) supports this presumption. The capillary

walls constitute the major fraction between 1/2-1/3 of the tissue. In the *rete* therefore the interstitial tissue would only represent between 1/9 and 1/6 of the cross-sectional area or of the volume.

The luminal surface of the endothelial cells especially in the venous capillaries showed microvillous processes similar to those present at the surface of mesothelial cells. Microvilli have been noted in the vessels of the pecten of the bird's eye and in the choroid *rete* of fish (Fawcett 1963) but also in mammalian vasculature such as in the rat Gasserian ganglion and testis (Gabbiani and Majno 1969) and in the dog pulmonary artery (Smith *et al.* 1971). The presence of microvilli in the capillaries of the *rete* may have an effect on the capillary exchange by making an increase in the capillary surface area and by producing an eddying flow (of cell free plasma?) along the endothelial surface.

The pinocytotic or plasmalemmal vesicles appeared to be similar to those of mammalian capillaries as to their size and concentration in the cells (Palade and Bruns 1968, Casley-Smith and Chin 1971). In addition to this microvesicular system the endothelial cells contained a separate system of vacuoles of considerably greater sizes as was also found by Dorn (1961) and Fawcett (1963). Such giant vacuoles have been described in mammalian endothelium by several investigators and are usually interpreted as representing a reaction to cell injury such as mechanical trauma, hypoxia, freezing, exposure to hyperosmotic solutions *etc.* (Luse and Harris 1960, Hoff and Gottlob 1967, Tsao and Spaet 1967). Different theories have been proposed for the genesis of these vacuoles: Mitochondrial swelling, autolysis of cytoplasmic content or dilatation of endoplasmic reticulum. However, it cannot be excluded that giant vacuoles may originate in a more normal way from plasmalemmal invaginations or more probably from a fuse of cell processes or marginal folds impounding droplets of luminal fluid which then move towards the cell center as clear vacuoles. The pictures shown in Fig. 12-14 are suggestive of such processes resulting in the formation of these vacuoles. If therefore this vacuolization of the endothelial cells actually reflects an *in vivo* phenomenon, it might represent a special transport activity which could play a significant role in the transcapillary exchange.

The width of the intercellular clefts were in the range of 100-300 Å. It was an almost constant finding that the clefts showed narrow regions. These narrow regions, most probably representing the junctions, could extend for 100-400 Å in depth. In the vicinity of these regions special junctional structures could be observed such as desmosomes with bundles of cytoplasmic fibrils converging on it—similar to those described by Farquhar and Palade (1963) and by Kelly (1966) in epithelia. One could also observe dense plaques which were located at one or both poles of the desmosomes, most probably representing attachment devices. In the structural studies of the retinal vessels earlier performed (Dorn 1961, Fawcett 1963, not quoted by Bendayan *et al.* 1974) the junctional lumina were always seen to be obliterated by such plaques. This finding would indicate these structures to represent continuous attachment bands running along the junction in a direction parallel to the free surface of the endothelium (*zonulae occludentes*). In the present study the major fraction of the junctions in the perfusion fixated *retina* as well as in the immersion fixated *retina* had patent channels with an average diameter of 110-120 Å. We would therefore suggest the existence of *maculae* rather than *zonulae occludentes* in the retinal endothelial cell.



tions. Attention should also be called to the detection of fine granulated less dense material in many junctions. If transcapillary transport actually takes place through the intercellular clefts we would have to consider that the diffusional resistance of this matrix consisting mainly of hyaluronic acid (Laurent 1970) may be different from that of water.

The basement membranes contained numerous microfibrils and often appeared as a layered structure. In general the basement membranes of capillaries have been described as having an homogenous structure. However the presence of distinct zones of different translucency has been described in intestinal capillaries of rats (Clementi and Palade 1969) and in lung capillaries of rabbits (Hovig *et al* 1971) after a treatment with EDTA sufficient to give formation of edema. In our studies no signs of edema could be detected not even in those preparations which had been perfused with Ringer solutions prior to the fixation.

The structure of the endothelial cells of the arterial capillaries were found to differ essentially from that of the venous endothelial cells. In the arterial vessels the endothelial cells were thick marginal folds were lacking and they had a continuous plasmalemma. The venous endothelium was more attenuated the number of pinocytotic vesicles was considerably smaller numerous marginal (cytoplasmic) folds could be detected and the plasmalemma was provided with clustered fenestrae. Thus the arterial capillaries seemed to be very similar to those of mammalian skeletal muscles whereas the venous vessels resembled some types of visceral capillaries in mammals as for instance those of the intestinal mucosa (Bennett *et al* 1959).

This dimorphism between the two sets of capillaries in the *rete* indicate the permeability properties of their walls to be significantly different. Intestinal capillaries have been shown to be more permeable than those of skeletal muscles the former allowing molecules of larger sizes to penetrate (Vogel and Strocker 1967) and having higher filtration coefficients (Folkow *et al* 1963). It appears reasonable to presume therefore that the arterial capillary walls will represent the greatest resistance to transcapillary diffusion in the *rete*.

According to the theory generally accepted the diffusion of water soluble molecules across the capillary walls takes place through the intercellular clefts (Landis and Pappenheimer 1963). Whether the junctions of skeletal muscle capillaries actually have patent gaps or not has been discussed (Karnovsky 1968, Bruns and Palade 1968 a). According to Karnovsky most of the junctions do represent open channels with a minimal width of 40 Å.

The fact that visceral capillaries are more permeable than capillaries of the continuous type both types having junctions with nearly the same structure and dimensions may indicate that the fenestrae represent an additional pathway to transcapillary transport. In fact Bruns and Palade (1968 b) and Karnovsky (1968) have shown that a considerable fraction of peroxidase or ferritin molecules appears to pass through the fenestrated regions of the endothelium. However the number of fenestrae in the venous capillaries of the *rete* was found to be significantly smaller than what has been observed in mammalian visceral capillaries. In for instance the renal peritubular capillaries the fenestrae may take up as much as one fifth of the total area (Frederick 1968). Accordingly then the venous capillaries in the *rete* could perhaps be expected to be less permeable than mammalian intestinal capillaries but more permeable than mammalian skeletal muscle capillaries.

These considerations about the permeability properties of the observed capillaries are

of course highly speculative. The structure of the capillary walls as it appears in the sections may thus not reflect the situation *in vivo*. Essential changes in structure—qualitative as well as quantitative—may take place during the fixation.

The present studies indicate the capillary endothelial cells to be highly differentiated ones. The endothelial cells of the *rete* were seen to contain a system of vacuoles as well as one of pinocytotic vesicles. These findings could indicate the participation of the endothelial cells in transcapillary transport. While transcellular transport by vacuoles may be a speculative hypothesis, the existence of transport through pinocytotic vesicles seems to be well established. Macromolecules such as ferritin ( $r=55 \text{ \AA}$ ) have been shown to be transported in such a way, indicating that the micro-vesicular system of endothelial cells may represent the large pore system of non fenestrated capillaries and lacteals (Bruns and Palade 1968 b; Dobbins and Rollins 1970; Casley Smith and Chun 1971). It has also been established that the major transcapillary transport of water in the *rete* takes place through the endothelial cells (Stray Pedersen and Steen 1975).

Bendayan *et al.* (1974) have made very interesting suggestions about the morphological and functional significance of the pinocytotic vesicles observed in capillary endothelial cells. These investigators used fixatives containing dimethyl sulphoxide and acrolein—substances which are known to contribute to good preservation of membrane structure (Sandborn 1966 and 1970). They assumed the vesicles (and vacuoles?) which appear as isolated structures on micrographs to represent sectioned parts of tortuous channels or tubules which are continuous throughout the cell. According to these investigators alternate widening and narrowing of such tubules may indicate that they exhibit some sort of peristaltic activity. It should be emphasized that a transcapillary transport through such a system of contractile tubules may be considerably more rapid and require less metabolic energy than transport via pinocytotic vesicles. It is also noteworthy that the diameter of the dilated parts of the tubules found by these investigators could be as great as  $1300 \text{ \AA}$ , a value which conforms precisely to the pore dimensions calculated on the basis of permeability data of the retial capillaries (Stray Pedersen and Steen 1975).

As seen from Table I the tissue content of  $K^+$  is significantly smaller and the content of  $Na^+$  significantly higher than that of homologous blood cells and human erythrocytes. The ratio between the concentrations of  $K^+$  and  $Na^+$  was found to be close to unity. As for  $Ca^{++}$  and  $Mg^{++}$  the content of these substances is very similar to that of homologous cells but considerably higher than that of human red cells which are extremely poor in  $Ca^{++}$ . These values for the ionic content of retial tissue however should be considered as rather rough estimations since no attempts were performed in order to estimate the fraction of intra-vascular fluid which might not be removed by squeezing and/or air inflation of the organ. The significance of the presented ionic concentration of retial tissue is not clear. If the endothelial cells are actually so poor in  $K^+$  one would expect the electrochemical potential of their membranes to be smaller than and the permeability properties perhaps essentially different from that of other cell membranes.

Studies on the permeability properties of the retial capillaries have been based on measurements of the exchange between the arterial and venous system (Stray Pedersen and Steen 1975; Stray Pedersen 1975). The actual barrier would therefore comprise two structurally

different capillary walls as well as the interposed tissue elements. As seen from Fig. 4 this barrier would vary considerably in thickness as well as in composition along the circumference of neighbouring vessels. At the levels of the angular interstices the arterio-venous barrier may have a thickness of several  $\mu$  consisting mainly of layers of interstitial tissue and cells. A transcapillary transport of any significance through these regions would therefore seem very unlikely. The other extreme is represented by those regions offering minimum resistance to transcapillary diffusion in which the barrier consists of following layers only: Arterial endothelium, arterial and venous basement membranes (partially fused) and venous endothelium. Such regions however are very unfrequently encountered and represent maximally 5% of the total exchange surface. In most places a pseudopodium of a pericyte and smaller amounts of interstitial tissue and fluid are seen to be imposed between the basement membranes. It seems reasonable to suggest that the major part of the transcapillary exchange takes place across this part of the barrier which comprises about 50% of the circumference of the capillaries. In conclusion then the "functional barrier" in the *rete mirabile* would consist of two outer layers consisting of arterial or venous endothelium with their basement membranes and a middle adventitial layer of a thickness of about 1  $\mu$ m. The average thickness of this "functional barrier" was found to be 1.6  $\mu$ m, the area constituting about 50% of the total capillary surface area.

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## Integrated Somatomotor, Cardiovascular and Gastrointestinal Adjustments Induced from the Cerebellar Fastigial Nucleus

By

BJÖRN LISANDER and JAN MARTNER

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### Abstract

LISANDER B and J MARTNER *Integrated somatomotor cardiovascular and gastrointestinal adjustments induced from the cerebellar fastigial nucleus* Acta physiol scand 1975 94 358-367

Behavioural cardiovascular and gastric responses induced by fastigial stimulation were observed in conscious cats with gastric fistulas indwelling fastigial electrodes and arterial catheters. Fastigial stimulation elicited oral behaviours *e.g.* grooming and chewing together with tachycardia and pressor responses while gastric motility was unaffected in most cases as was gastric hydrochloric secretion. In subsequent experiments on the anesthetized animals it was found that the same fastigial area could suppress the intestino-gastric inhibitory reflex. Fastigial influences on small intestinal motility were investigated in anesthetized cats, well recovered from surgical isolation of intestinal loops whose motility could therefore be recorded without

Fastigial stimulation either depressed or did not influence ileal motility before laparotomy but after this procedure excitatory responses were uniformly recorded. This reversal is explained by a fastigial suppression of inhibitory intestinal reflexes elicited by the laparotomy.

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Stimulation of the rostral fastigial pole elicits a powerful pressor response (Achari and Downman 1969; Miura and Reis 1969) and in unanesthetized cats or rats complex behavioural responses including grooming, biting and eating (Reis, Doba and Nathan 1973; Burnston, Potolicchio and Miller 1973; Ball, Micco and Burnston 1974). This fastigial pressor area may also modify several autonomic mechanisms controlling gastrointestinal motility in anesthetized cats (Lisander and Martner 1974, 1975). The question arises which gastrointestinal influences that are elicited in unanesthetized cats since cerebellar autonomic responses can differ markedly in conscious and anesthetized animals (Rasheed, Manchanda and Anand 1970). Second are the behavioural responses associated with specific patterns of gastric function in terms of motility and secretion? Are the changes in behaviour secondary to the visceral adjustments? To elucidate such problems experiments were performed in conscious cats where chronically implanted fastigial electrodes could be stimulated and the effects on gastric volume or secretion could be followed.

Concerning intestinal motility the fastigial influence is mainly excitatory in anesthetized cats. It has been proposed that this reflects a suppression of the sympathetic intestino-intestinal

inhibitory reflex (Lisander and Martner 1974 Martner 1975) a term here also used for inhibitions elicited by the inevitable trauma from opening the abdomen. If so, then quite different fastigial effects on intestinal motility might be revealed if instead a less traumatic abdominal preparation is used. Therefore the second part of this study was devoted to atraumatic intestinal motility recording on isolated intestinal loops with the ends emerging through the abdominal wall in animals previously operated and completely recovered.

## Methods

The experiments were carried out on 17 animals of either sex. All operations were performed under Nembutal® (Abbott) anesthesia (30–40 mg/kg b.w.) with endotracheal intubation and aseptic precautions. Before the animals ate postoperatively saline was given subcutaneously. A mixture of streptomycin and penicillin (Streptocillin® Novo 0.5 g + 00 000 IU) was injected daily for 5–14 days. After the various operations the animals recovered rapidly and appeared undisturbed within one week. All cats except two exceeded their preoperative weight at the end of the experimental period.

### *Gastric fistula operation*

The abdomen was opened in the midline. An incision was made in the stomach close to the major curvature. A plexiglass cannula (inner diameter 14 mm) was fitted into the opening and the end of the cannula was brought out through a stab wound on the left side of the abdomen. The midline opening was closed in layers. The animals were allowed to eat on the second or third day after the operation. These animals were three weeks to two months later implanted with fastigial electrodes and an arterial catheter.

### *Isolation of an intestinal segment (6 cats)*

The abdomen was opened in the midline. A segment of jejunum or ileum 10–15 cm long was isolated keeping the mesenteric nervous and vascular supply intact. The rest of the intestine was anastomosed end to end. The ends of the isolated segment were brought out through two stab wounds on the right side of the abdomen. The serosa was sutured to the skin and the midline opening was closed in layers. The animals were allowed to eat from the day after the operation.

### *Implantation of a arterial catheter and cerebellar electrodes (8 cats)*

The neck was incised and the right carotid artery cannulated by a silicon rubber catheter (Silastic, Dow Corning, inner diameter 0.76 mm) with the tip in the aortic arch. The other end of the catheter was connected to a P33 AC transducer resting on a Grass polygraph.

Then the head of the animal was placed in a Horsley-Clarke apparatus. The skin was incised and trephination was made in the skull over the cerebellum. Electrodes made from teflon coated platinum-iridium wire (Medwire Corp. wire diameter 0.3 mm) were inserted bilaterally in the fastigial pressor areas at an angle 15° posterior to the vertical plane. The optimal location of the electrodes was judged by the blood pressure response to test stimulations. They were then fixed in position by dental cement and connected to a microplug provided with a stainless steel hook. The plug was hooked to the bone through a burr hole and fixed with cement. The hook was later used as the positive electrode. The free end of the blood pressure catheter was drawn under the skin to the opening in the scalp. The catheter was connected to a small valve (see below) screwed to the bone after which the skin wounds were closed. The valve and the microplug projected through the skin. The arterial catheter was kept open by daily flushings with heparin solution. — In three additional animals only arterial catheters were implanted.

### *Observations in the anesthetized animals*

The animals' behavioural responses to fastigial stimulation were observed in a box with a one-way mirror at least 5 days after the electrode implantation. A polyethylene tube with a connector was screwed to the valve on the head. The valve was opened by this procedure. The arterial pressure was recorded by a Grass P33 AC transducer.

Gastric juice was collected by rinsing the stomach with 10 ml of saline as a short tube through the gastrotomy. Titration was done with 0.1 N NaOH with phenolphthalein as the indicator. To increase gastric

secretion, histamine chloride (Virum) was injected subcutaneously. Gastric volume was recorded by the balloon method and a Grass force transducer with the animal kept in a small cage. The intraluminal pressure was kept at 5–10 cm H<sub>2</sub>O (Lisander 1975; Lisander and Martner 1975).

#### *Acute experiments on anesthetized animals*

All cats were chloralosed (50–60 mg/kg b.w. i.v.). To eliminate any artifacts due to somatomotor changes diallyl-bis nor toifenine-dichloride (Alloferin® Roche) 0.1 mg/kg i.v. was given. Artificial respiration was then maintained. In the group with chronic implanted fastigial electrodes stimulation was performed via these electrodes while electrodes were acutely inserted in the other cats. Square wave pulses at intensities from 0.0– to 0.2 mA were used (in one cat 0.6 mA). Pulse duration was usually set at 1 ms and the impulse frequency at 50 Hz (Lisander and Martner 1975).

Gastric motility was recorded as described above though the intragastric balloon was inserted via the esophagus in the cats without a gastric fistula. Intestinal motility was recorded as pressure changes in a balloon inserted into the isolated intestinal loop. Distending pressures (100–200 cm H<sub>2</sub>O) could also be applied to this loop in order to elicit an intestino-gastric inhibitory reflex.

#### *Histological procedures*

After the acute experiments the heads of the animals were perfused by saline followed by a 10% solution of formaldehyde. The cerebellum and adjacent brainstem structures were then paraffin embedded, sectioned in slices of 15 µm and stained by the Nissl technique. In some experiments the cerebellum was frozen sectioned.

## Results

### *1 Conscious cats with fastigial electrodes*

Cats with a gastric fistula were later implanted with chronic fastigial electrodes thus allowing for simultaneous observation of behaviour and gastric motility or acid production. The results from these animals are summarized in Table I.

*Behaviour.* Although all electrodes gave marked pressor responses, some differences in electrode site could not be avoided. However, the similarities between behaviour patterns in different cats were remarkable, although the stimulation thresholds could vary considerably between the cats. If the electrode tip was placed in the rostral ventromedial fastigial pole 0.02 mA was sufficient to elicit responses while in one cat where the electrode tip was placed 0.5 mm caudally to this area, 0.5–0.6 mA was necessary to elicit the same response. The character of the responses was determined partly by stimulation intensity. Thus, at

TABLE I. Observations from 7 cats during fastigial stimulation. Abbreviations and signs: E Eating, G Grooming, I No influence, – Inhibition. Gastric motility refers to the situation in the unanesthetized animal and the influence on the IGIR (intestino-gastric inhibitory reflex) was tested in the terminal experiment.

Cat no	Behaviour	Gastric motility	Influence on IGIR
1	E, G	0	–
2	G	0	–
3	G	–	–
5	G	–	–
6	G	0–	I
7	G	0	–
9	G	I	–

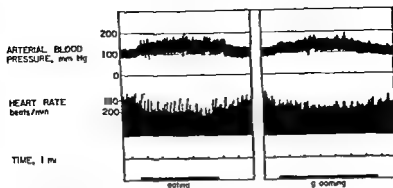


Fig. 1. Conscious cat 3.0 kg with an indwelling arterial catheter. Note that spontaneous eating and grooming are associated with increased blood pressure and heart rate.

threshold intensity sporadic licking of the mouth appeared. If the stimulation was prolonged the cat began licking its fur. At slightly increased intensity the cat usually pulled at the fur with its front teeth. The grooming in most cases started with either front paw and then progressed to other regions if stimulation was prolonged. With increasing intensity the grooming became more and more vigorous. The responses generally started immediately after the onset of stimulation and ceased within five seconds after the stimulus. In the animals reported here they were entirely reproducible throughout the experiment and could be repeated in a later test.

The animals had free access to water, milk and minced fish together with a few large rubber corks. Only in one cat stimulation caused eating together with grooming. Very often, especially at higher intensities, the animals interrupted their grooming and started biting at the cardboard covering the bottom of the box. Pieces were torn away but not ingested. If instead a rubber cork was located very near the animal could take a few bites on it.

No clear signs of aggression were noted and the animals could be handled even during intense stimulation without danger. They only interrupted their stereotype behaviour for a few seconds if the box was opened and they were touched during stimulation. If another cat was put into the cage the stimulated animal behaved as before during stimulation even if it displayed hostility towards the intruder between stimulations.

The somatomotor response pattern was thus stereotyped, but performed in a well coordinated fashion with no ataxia or other motor disturbances. No signs that the stimulations might be irritating or painful, such as growling, hissing or attempts to escape, were noted.

The thresholds for the blood pressure increase and for the above described behaviour were identical whether current or impulse frequency was varied. Spontaneous grooming was paralleled by marked increases in blood pressure and heart rate (Fig. 1). The same observation was repeatedly made in three other cats implanted with arterial catheters only.

*Gastric motility.* While stimulation in conscious cats caused pronounced effects on blood pressure and behaviour changes in gastric motility were seldom demonstrated. In only three cats prompt inhibitions of gastric tone were elicited resembling adrenergically



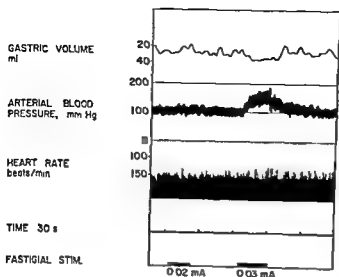


Fig 2 Conscious cat 27 kg  
Fastigial stimulation (50 Hz 1 ms)  
0.02 mA produces no response  
while 0.03 mA blood pressure  
rise and inhibition of gastric  
motility are elicited together with  
grooming and gnawing on card  
board

relaxation in acute experiments on anesthetized cats (Lisander and Martner 1975). This response had the same threshold as the blood pressure response (Fig 2).

**Gastric HCl secretion** The results from cats tested for fastigial influence on gastric HCl production are shown in Fig 3. The mean prestimulatory values are somewhat higher than basal HCl secretion in cat 0.01–0.05 mEq per hour (Emås 1960). Fastigial stimulation did not change HCl secretion while subsequent submaximal histamine administration increased acid output.

## 2 Anesthetized animals

a) **Gastric motility** In subsequent experiments performed on the anesthetized animals fastigial stimulation produced either slight inhibitory (4 cats) or biphasic gastric responses (1 cat). However following laparotomy the only response pattern obtained was that of

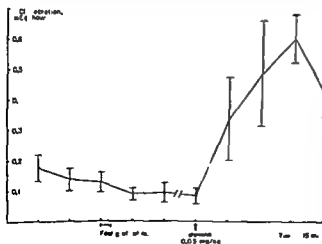
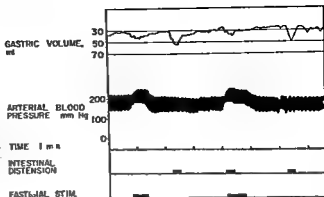


Fig. 3 Compiled results from recordings of HCl secretion in 5 cats taken at 15 min interval. Bars indicate S.E. After the intersection  $n=4$  as one cat was omitted from the histamine test. Note that fastigial stimulation (30 s each minute for 5 min) does not produce any significant change in HCl secretion.

Fig. 4 Cat 3.1 kg with isolated ileal segment. Chloralose anesthesia, Alloferin® 0.1 mg/kg b.w. i.v. artificial respiration. The vagal nerves cut and the distal ends stimulated (6 Hz, 1 ms 8 V). Ileal distension produces gastric relaxation. Note that a concomitant fastigial stimulation (50 Hz, 1 ms 0.05 mA) which in itself produces relaxation, totally abolishes the gastric response to intestinal distension.



excitation. The latter kind of response was still present after vagotomy provided that the distal ends of the cut vagal nerve were stimulated but disappeared following guanethidine administration. This denotes fastigial inhibition of the intestino-gastric inhibitory reflex (Lisander and Martner 1975).

b) *Gastric motility responses to intestinal distension*. If a distending pressure was applied to the isolated intestinal loop an intestino-gastric inhibitory reflex could be elicited provided a vagal tonic activity was present. Concomitant fastigial stimulation abolished or markedly reduced this sympathetically mediated reflex (Fig. 4) although fastigial stimulation alone had

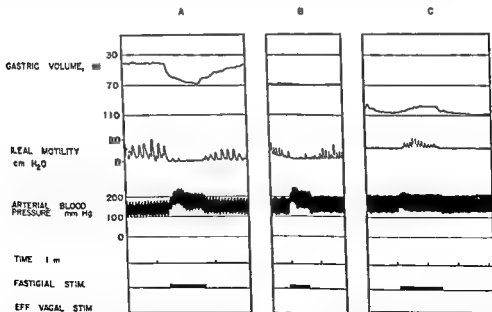


Fig. 5 Cat 2.5 kg, isolated ileal segment. Chloralose anesthesia. A Fastigial stimulation (50 Hz, 1 ms, 0.1 mA) produces inhibition of ileal and gastric motility. B After bilateral vagotomy the ileal inhibitory response is abolished. C Following laparotomy and efferent vagal stimulation (5 Hz, 1 ms, 5 V) both the stomach and the ileum display excitatory responses.

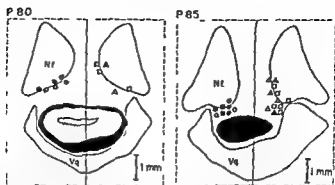


Fig 6 Cerebellar frontal sections 8 and 8.5 mm posterior to the interaural line. Results from conscious cats are shown to the left and responses from anesthetized cats to the right. Electrode sites in one cat at P 7.5 and in another at P 9.0 are projected to P 8.0 and 8.5 respectively. Filled circles: Oral behaviour. Open circles: No influence on gastric motility in spite of oral behaviour. Filled squares: Inhibition of gastric motility. Open squares: Inhibition of the intestino-gastric inhibitory reflex. Open triangles: Inhibitory intestinal responses before laparotomy. Filled triangles: Excitatory intestinal response after laparotomy. Nf: Nucleus fastigius. Vq: Ventriculus quartus.

a slight relaxatory influence on the stomach. Even pronounced fastigially induced gastric relaxations could be changed into excitations following laparotomy as illustrated in Fig 5.

*c) Ileal motility.* In intact cats fastigial stimulation did either not influence ileal motility or, as depicted in Fig 5 panel A, produced an inhibition of spontaneous ileal contractions still present after vagotomy (panel B). However, after laparotomy, depressing ileal motility, fastigial stimulation enhanced the motility in all 4 cats during the entire stimulation period (panel C). The latter fastigial response did not require intact vagal nerves but could be blocked by guanethidine.

*d) Jejunal motility.* Two cats had isolated jejunal segments. As in the other cats, jejunal distension caused gastric inhibition which was suppressed by concomitant fastigial stimulation. Motility in the jejunal segments was either unaffected or slightly inhibited by fastigial stimulation. Laparotomy depressed jejunal peristalsis but only transiently and no clear excitatory responses to fastigial stimulation were present after this procedure.

#### Electrode positions

Histological examination confirmed that the effects were elicited from the fastigial pressor area, i.e. the ventromedial rostral fastigial pole. In Fig. 6 the different kinds of responses from the conscious cats are shown on the left. Electrode sites and the character of the responses from the experiments on the anesthetized cats are depicted to the right.

#### Discussion

Stimulation of the anterior pole of the fastigial nucleus elicited in the conscious cats grooming, biting, chewing and occasionally eating, confirming findings by Reis *et al* (1973) and Bernston *et al* (1973). Similar oral responses have also been observed in rats (Ball *et al* 1974). Zanchetti and Zoccolini (1954) reported that fastigial stimulation could

trigger sham rage in decorticated cats Reis *et al* (1973) also reported aggressive behaviour as a result of fastigial stimulation which however was not observed in the present study. When they were exposed to fastigial stimulation they showed little interest in their environment and started instead gnawing and tearing at the nearest suitable object in a *per se* well coordinated manner but did not as is typical for hypothalamically induced feeding responses explore the surroundings in search of appropriate goal objects. These present cerebellar responses took place only during stimulation whereas fastigial feeding responses are less stimulation bound (cf Ball *et al* 1974). Actual ingestion of food during fastigial stimulation was only seen in one of the animals.

Even though stimulation thresholds and electrode positions differed somewhat between the cats the similarities in the animals concerning their fastigially induced responses were striking. Thresholds for the autonomic and behavioural responses were 2.5-3.0 mA suggesting that the stimulated fastigial pressor area might induce an integrated pattern of autonomic and somatomotor adjustments. This possibility is also supported by the fact that spontaneously elicited grooming behaviour was paralleled by similar cardiovascular increases in blood pressure and heart rate thus resembling the responses to fastigial stimulation. A conspicuous finding in the unanesthetized animals was the occurrence of specific gastric motility changes associated with the oral behavioural responses to fastigial stimulation somewhat surprising since experiments on anesthetized animals have demonstrated that the fastigial nucleus is quite potent in modifying gastric motility (Martner 1975). Further none of five unanesthetized cats tested for gastric motility responses and all displaying grooming, biting and chewing showed a significant increase in HCl secretion in association with these stimulations. Had a truly 'emotional' response been evoked in connection with these fastigial responses one would have expected a decrease in gastric secretion to take place since it is known that anticipation of eating emotional in nature or hypothalamic stimulation etc can increase gastric secretion (cf Vasey and Mason and Nelsen 1969).

This lack of regularly occurring stimulation bound gastric responses in the unanesthetized animals may suggest that the particular grooming and chewing responses to fastigial stimulation is not primarily elicited by e.g. afferent impulses from the oral cavity involved in feeding behaviour. On the other hand the mentioned responses may be part of various well integrated behavioural responses to stimulation of the fastigial nucleus which also induce complex cardiovascular and gastrointestinal adjustments. It is possible that these phylogenetically old cerebellar parts control not only the autonomic responses but also complex behavioural patterns normally initiated from higher brain centres. Further studies are needed before such a hypothesis can be tested.

When the same animals were used for acute experiments the fastigial stimulation caused the same gastric motility responses (cf Martner 1975) as long as the animals were not exposed to strong vagal stimulation. Inhibitory or occasionally biphasic responses. The former by a direct vagal inhibition of motility and the latter by a combination of vagal inhibition and an augmented vagal cholinergic activity (Lisander *et al* 1974). Soon as the animals were exposed to laparotomy there was a marked increase in gastric motility.

sympathetic intestino-intestinal (intestino-gastric) inhibitory reflexes fastigial stimulation elicited only excitatory gastric responses. These were due to a fastigial suppression of the mentioned spinal inhibitory reflexes induced by laparotomy (*cf* Lisander and Martner 1975).

In the animals with chronically isolated intestinal loops pronounced adrenergically mediated, intestino-gastric inhibitory reflexes could be induced by distension of these loops (*cf* Piercy and van Liere 1926 Youmans 1968). Fastigial stimulation substantially reduced these reflex inhibitions of gastric motility. As long as the abdominal cavity was left intact fastigial stimulations alone produced as mentioned gastric inhibitory or biphasic responses and, in the chronically isolated intestinal loops either no appreciable response or motility inhibition. After laparotomy only excitatory responses were encountered in the ileal loops while the jejunal loops (two cats) showed no or very weak excitatory responses. This is in agreement with the effects of fastigial stimulation in acute experiments on laparotomized cats, where gastric and ileal responses are uniformly excitatory while the jejunum displays a mixed response pattern (Lisander and Martner 1974 Martner 1975). The present findings thus support the view that the fastigial nucleus and hence the cerebellum, exerts control of gastrointestinal motility predominantly though not only by suppression of the sympathetically mediated intestino-gastric inhibitory reflexes whenever such spinal reflex arcs are activated (Lisander and Martner 1975 Martner 1975). The weaker effects on the jejunal loops might simply be due to a less intense adrenergic impact on this part of the gut (Kewenter 1965).

The present results concerning neurogenic gastrointestinal control also demonstrate the importance of the experimental situation for the outcome of the effects. In studies of motility in general abdominal surgery is often inevitable thus eliciting the spinal inhibitory reflexes. These can, in turn, be suppressed by structures of the medullary "depressor area" (Johansson, Jonsson and Ljung 1965) and from the cerebellum according to the present results and probably from other parts of the central nervous system as well. Evidently it is important for the correct interpretation of results concerning nervous control of gastrointestinal function first to define clearly the experimental circumstances involved. For example depending upon whether or not the preparation employed has led to an activation of the spinal sympathetic inhibitory reflexes results of different experimental studies may be strikingly different and apparently contradictory.

Thus, the present study has shown that restricted parts of the cerebellar fastigial nucleus can induce well-integrated behavioural patterns associated with complex adjustments of the cardiovascular and gastrointestinal systems. Evidence is accumulating to indicate that the cerebellum, besides its well-known coordination of simple somatomotor movements controls also visceral and emotional functions as well as certain complex behavioural patterns (*cf* Zanchetti and Zoccolini 1954 Bernston *et al* 1973 Reis *et al* 1973 Dow 1974 Ball *et al* 1974). The results of the present study are in agreement with such a concept.

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## Effects on Gastric Motility from the Cerebellar Fastigial Nucleus

By

Björn LISANDER AND JAN MARTNER

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### Abstract

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LISANDER B and J MARTNER *Effects on gastric motility from the cerebellar fastigial nucleus*  
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In acute experiments on chloralosed cats gastric motility, blood pressure and heart rate were investigated for influences exerted by the fastigial nucleus. Besides pressor responses, fastigial stimulation could produce either gastric excitation or relaxation and the background of these responses was analysed by selective nerve sectioning and administration of suitable autonomic blocking agents. Suppression of prevailing gastric motility was found to be mediated mainly by increased discharge in adrenergic nerve fibres but also by adrenal catecholamine release. — Gastric excitation could be induced in three different ways: first via increased activity in vagal cholinergic fibres, second by fastigial suppression of the vago-vagal non-adrenergic relaxatory reflex. In addition, when laparotomy or other noxious abdominal stimuli had induced inhibitory gastric reflexes, the consequent sympathetic discharge could be suppressed by fastigial stimulation resulting in enhanced gastric motility. — The importance of background activity in the various nervous pathways for the fastigially induced gastric responses is discussed.

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Central nervous control of gastric function has been extensively investigated and is well documented. Gastric motility may be influenced from various parts of the central nervous system, such as certain cortical structures, hypothalamus and brain stem (for details see Thomas and Baldwin 1968). Also the cerebellum seems to be involved in the control of gastric function, although only few studies have been carried out to elucidate this matter. Bard *et al.* (1947) reported that motion sickness in dogs is abolished by extirpation of the uvula and nodulus. Wolfe (1969) showed that gastric ulceration sometimes follows restricted cerebellar lesions in cats. Gastric motility can be influenced from the anterior lobe in rabbits (Ban *et al.* 1956) and from the vermis and the fastigial nucleus in cats (Manchanda, Tandon and Anuja 1972).

In general, most visceral functions can be modified from the cerebellum and the fastigial nucleus seems to be especially potent in giving autonomic responses. Thus, powerful pressor responses can be elicited by electrical stimulation of the ventromedial part of the rostral fastigial pole (Achari and Downman 1969; Miura and Reis 1969). However, this area can influence also other autonomically innervated organs, e.g. the intestinal tract (Lisander and Martner 1974; Martner 1975 a) and the urinary bladder (Martner 1975 b). The findings

presented in these studies indicated that an augmentation of intestinal motility could be induced by a fastigial suppression of the adrenergically mediated intestino-intestinal inhibitory reflex. This reflex inhibits motility throughout the gastrointestinal tract following distension of any part of the intestine (cf Pearty and Liere 1926 Youmans 1968). Besides intestinal distension any noxious stimulus to the abdomen including laparotomy depresses gastrointestinal motility and this is here included in the term intestino-intestinal inhibitory reflexes.

Studies on the stomach offer certain advantages in the further investigation of cerebellar influences on this type of reflex which below will be referred to as the intestino-gastric inhibitory reflex as far as the stomach is concerned. In the first place it is possible to record gastric motility without laparotomy a procedure which inevitably elicits the intestino-gastric inhibitory reflex. Second background cholinergic tone which is necessary for demonstrating this reflex (Jansson and Martinson 1966 Jansson and Lisander 1969) is much easier to control in the stomach e.g. by graded efferent vagal stimulation than in the intestines which usually display more irregular intrinsic activity. The present study was undertaken to investigate the fastigial influence on gastric motility and if such an influence could be demonstrated to analyse the nervous mechanisms involved.

### Methods

43 cats of either sex deprived of food for 24 h were used for the experiments. After induction with ether anaesthesia was maintained by i.v. administration of chloralose 50–60 mg/kg b.w. For stereotaxic stimulation the head of the animal was fixed in a Horsley Clarke apparatus. Following trephination and partial removal of the tentorium, sharp monopolar stainless steel electrodes were inserted perpendicularly to the stereotaxic horizontal plane. Square wave pulses were delivered by a constant current stimulator at intensities varying from 0.05 to 0.3 mA corresponding to a voltage range of 1–4 V. Pulse duration was set at 1 ms and the impulse frequency usually at 50 Hz after recordings of the frequency-response relationship at 10 to 60 Hz. Decerebration was performed at the intercollicular level either with a blunt spatula or by using high frequency electric coagulation by a stereotaxically inserted needle electrode.

After each experiment a small lesion was made around the cerebellar electrode tip by anodal direct current of 1 mA for 10 s. The head of the animal was then perfused with saline followed by a 10% solution of formaldehyde. In 1/3 of the cats the cerebellum and adjacent brain stem structures were paraffin embedded, sectioned in slices of 15  $\mu$ m and stained by the Nissl technique. In the rest of the material the cerebellum was frozen, sectioned and mounted without staining. In the latter way the precise location of the intercollicular lesion was determined as well.

Intestino-gastric inhibitory reflexes were elicited either by laparotomy or by repeated injections of 0.1–1 ml of acid (0.1 M HCl) or alkali (0.1 M NaOH) through the abdominal wall. The vagal nerves were dissected free in the neck and often cut in the course of the experiment. The distal ends were then placed on bipolar ring electrodes for graded efferent stimulation. When instead the vago-vagal gastric relaxatory reflex was studied one vagal nerve was left intact but the other one cut with the central end mounted on a bipolar electrode (for details see Jansson 1969 a) for afferent stimulation (10–50 Hz, 1–2 ms and 6–15 V). In other cats arrangements were made for reversible blockade of vagal transmission by temporary nerve cooling at the neck level. Spinal cord transection was performed between  $C_6$ – $C_7$  or between  $C_7$ – $Th_1$ .

In cats subject to laparotomy adrenal secretions were eliminated by encircling ligatures around both glands. Adrenocortical substitution was then given by i.v. injection of hydrocortisone (Solu-Gluc® Erco) 10 mg/kg. To allow for graded baroreceptor stimulation in some experiments one carotid sinus region was partly isolated and via a polyethylene tubing connected to one of the femoral arteries. A sigma motor perfused on pump could change the pressure in the sinus with a wide limits pressure being measured from a side branch close to the sinus by means of a P23 AC transducer. In these experiments the contralateral sinus nerve and also the vagal nerves were sectioned thus eliminating other baroreceptor stations.



Arterial pressure was measured through a femoral catheter connected to a Statham P23 AC transducer writing on a Grass polygraph and heart rate was recorded by a Grass Tachograph unit. Gastric motility was measured as volume changes in a large intragastric waterfilled balloon inserted into the stomach via the esophagus. Via a large caliber tube the balloon was connected to a volume reservoir whose weight was continuously recorded by a Grass force displacement transducer FTO3. The water level was adjusted to obtain a constant intragastric pressure of 5–10 cm H<sub>2</sub>O (for details see Jansson 1969 a).

Gallamine iodide (Flaxedil®) 4 mg/kg b.w. i.v. or diallyl bis-nor toxoferine-dichloride (Alloferin® Roche) 0.1 mg/kg b.w. i.v. was often used to eliminate any artifacts due to somatomotor changes. Artificial respiration was then maintained by a respiratory pump. Pharmacological adrenergic blockade was induced by guanethidine (Ismelin® CIBA) 4–5 mg/kg b.w. i.v. in some cats in combination with phentolamine (Regitin® CIBA) 3 mg/kg b.w. i.v. and propranolol (Inderal® ICI) 0.5–1 mg/kg b.w. i.v. For cholinergic blockade atropine (atropine sulphate) 0.5–1 mg/kg b.w. i.v. or methyiscopolamine (Skopyl® Pharmacia) 0.015–0.03 mg/kg b.w. i.v. were used.

## Results

Fastigial stimulation was found to influence gastric motility in various ways. Background gastric tone, which to a great extent depends on the preparation procedures, was an important factor in determining the response pattern. Therefore the experiments were subdivided into different groups depending on the initial operation and subsequent experimental arrangements.

### 1 Fastigial influence on gastric motility in intact cats

Out of a total number of 23 cats in this group 11 displayed either increased or decreased gastric motility while the remainder showed a biphasic response pattern. In the latter group the sequence of the responses was always the same, *i.e.* an initial suppression followed by an increased gastric tone, even if the net response in some cats was dominated by inhibition and in others by excitation. If the latter group was added to those cats exhibiting uniform excitation and the former group included in the total number of cats displaying inhibition, each group contained about the same number of cats.

Gastric relaxations induced by fastigial stimulation were either abolished or substantially reduced by parasympathetic blockade. Thus atropine (1 cat) or vagotomy (4 cats) eliminated the response, while the latter procedure in 3 other cats substantially reduced suppression of gastric motility. If the distal ends of the cut vagal nerves were stimulated, gastric vagal tone could be reestablished artificially and it was then again possible to demonstrate fastigially induced gastric relaxation. After administration of the adrenergic blocking agent guanethidine (7 cats) the gastric relaxations were abolished in 3 cats but persisted in 4 cats though reduced and delayed in onset, suggesting a hormonal mechanism. The disappearance of gastric relaxation after adrenergic blockade is illustrated in Fig. 1. Spinal cord transection in 2 cats displaying a biphasic gastric response abolished the inhibitory phase while the excitation persisted. Surprisingly, in one other cat spinal cord transection failed to abolish gastric relaxation which was instead blocked by atropine.

Excitatory gastric responses were completely abolished by atropine (2 cats) or by vagotomy (4 cats) while spinal cord transection failed to do so (4 cats). In the spinal cats, however, the gastric contractions were abolished by administration of atropine or methyiscopolamine as is illustrated in Fig. 2. Also evident from this figure is the abolition of fastigially induced tachycardia.

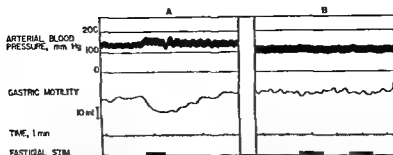


Fig 1 Cat 2.4 kg, right vagal nerve cut. Fastigial stimulation (50 Hz, 1 ms, 0.3 mA) induces a prompt gastric relaxation together with a blood pressure rise (panel A). After administration of guanethidine 4 mg/kg b.w. i.v., both the gastric and the blood pressure responses are abolished (panel B).

## 2. Fastigial influence on gastric motility in cats subject to laparotomy

Laparotomy invariably induced a profound adrenergically induced depression of gastric tone by the intestino-gastric inhibitory reflex. Independent of the type of fastigial response obtained before laparotomy the response pattern following this procedure was always that of excitation. The total number of cats in this group amounted to 14 if 5 cats receiving intra abdominal injections of acid or alkali (see later) were included. Bilateral vagotomy eliminated or reduced the excitatory responses to fastigial stimulation. However the responses could always be restored or even potentiated if the distal ends of the cut vagal nerves and the fastigial nucleus were concomitantly stimulated. Fig 3 clearly illustrates the importance of background vagal cholinergic activity. Fastigial stimulation in the absence of vagally induced gastric tone evokes no response while there is a substantial increase in gastric tone when fastigial stimulation is performed during concomitant vagal stimulation usually the more so the higher the frequency of vagal stimulation up to about 6 Hz. — If time was allowed to elapse after vagotomy stomach motility often returned without vagal stimulation.

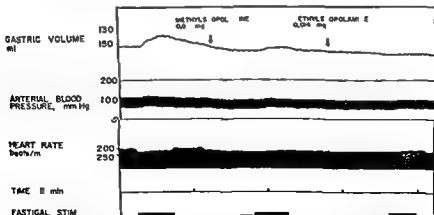


Fig 2. Cat 3.6 kg, the spinal cord transected between C<sub>7</sub> and Th<sub>1</sub>. Fastigial stimulation (50 Hz, 1 ms, 0.1 mA) produces an excitatory gastric response which is eliminated by subsequent administration of methylscopolamine.

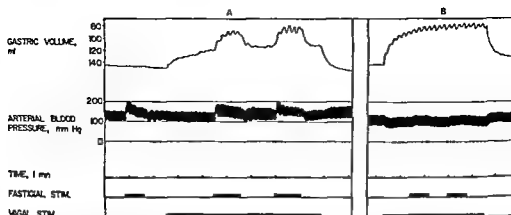


Fig. 3 Cat 3.1 kg vagotomized and laparotomized with adrenals ligated. A Fastigial stimulation (50 Hz, 1 ms, 0.2 mA) with and without efferent vagal nerve stimulation (6 Hz, 2 ms, 8 V). Note that gastric responses are only elicited against a background of vagal activity. B When adrenergic inhibitory discharge is blocked by guanethidine (4 mg/kg i.v.) even low frequency vagal stimulation (4 Hz) induces the same level of gastric excitation that required a combined vagal and fastigial stimulation in A. Moreover no fastigial effects can now be demonstrated.

and it was then again possible to enhance this motility by fastigial stimulation. Administration of acid (HCl) or alkali (NaOH) also induced a transient intestino gastric inhibitory reflex which could be abolished by guanethidine. When gastric tone was reflexly inhibited in this way fastigial stimulation again induced excitatory gastric responses evidently mediated by adrenergic mechanisms since they were eliminated by guanethidine (4 laparotomized cats and one injected with acid).

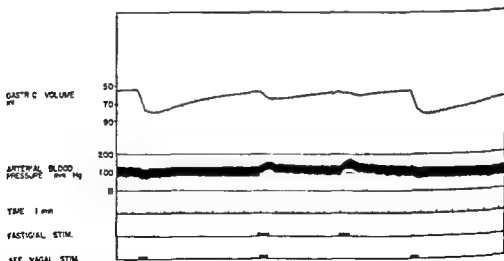
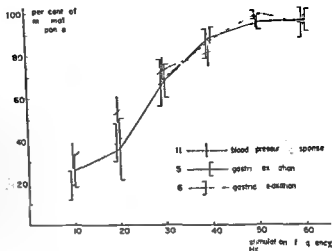


Fig. 4 Cat 3.2 kg adrenergic blockade (not complete which is evident from the remaining blood pressure rise) with guanethidine (5 mg/kg), phentolamine (3 mg/kg) and propranolol (1 mg/kg). Cholinergic blockade with atropine, 1 mg/kg. The vago-vagal relaxatory reflex is elicited by afferent vagal stimulation (14 Hz, 2 ms, 8 V) and produces a profound depression of gastric tone. Note that concomitant fastigial stimulation (50 Hz, 1 ms, 0.2 mA) inhibits this reflex.

Fig. 5 Frequency-response curves for fastigial stimulation compiled from 11 cats comparing blood pressure rise excitatory and inhibitory gastric responses. The excitations are in these cats due to suppression of the intestino-gastric inhibitory reflex while gastric inhibitions are produced by increased adrenergic discharge. Note that all 3 curves follow a similar course. Bars indicate S.E.



The excitatory gastric responses in laparotomized cats remained after decerebration (3 expts.)

The fastigially induced gastric effects were not secondary to baroreceptor reflexes since the motility responses were not influenced by artificially varied carotid sinus pressure in three animals.

### 3 Fastigial influence on the vago-vagal nonadrenergic gastric relaxatory reflex

Electrical stimulation of the central end of one cut vagal nerve with the other left intact regularly induced profound and longlasting gastric relaxations that persisted after both adrenergic and cholinergic blockade (Jansson 1969 a). In 7 out of 9 cats treated with atropine and adrenergic blockade (6 cats treated by guanethidine and 3 by spinal cord section) it was possible to inhibit this vago-vagal gastric relaxation by fastigial stimulation. The range of fastigial inhibition varied from merely changing the speed of the gastric relaxation to almost complete blocking of the reflex (Fig. 4). In this experiment fastigial stimulation *per se* induced a modest but prolonged gastric relaxation probably due to adrenal catecholamine release and if this relaxation is taken into account (third signal in Fig. 4) the fastigial suppression of the vago-vagal reflex seems to be nearly complete.

### 4 Stimulus-response relationships

The fastigial pressor response displays a gradually rising frequency-response curve from 10 Hz up to 50 Hz which in most experiments was the optimal frequency level. When compared with the frequency response curves for the fastigially induced gastric inhibitions and excitations respectively they show a conspicuous congruence in virtually all respects (Fig. 5). No difference in threshold could be observed for these three types of responses if instead current was varied.

### 5 Response cerebellar structures

Almost all gastric responses were elicited from the fastigial pressor area. As seen from Fig. 6 showing three frontal sections 8.85 and 9 mm posterior to the interaural line the signs

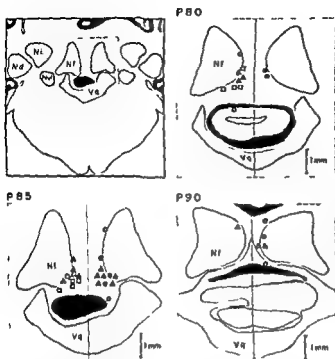


Fig. 8. Frontal sections 8.85 and 9 mm posterior to the interaural line. For the sake of clarity the same types of gastric responses are always gathered on the same side of the drawings. Open circles: No response. Open triangles: Gastric relaxations. Open squares: Suppression of intestino-gastric inhibitory reflex. Filled triangles: Gastric excitatory responses. Filled dots: Suppression of the vago-vagal inhibitory reflex. Nf: nucleus fastigius. Nl: Nucleus interpositus. Nd: Nucleus dentatus. Vq: Ventriculus quartus. Nvl: Nucleus vestibularis lateralis.

representing effective stimulation points are localized in the rostral ventromedial part of the fastigial nucleus and adjacent cerebellar white matter anatomically corresponding to the fastigial pressor area. In only 3 cats clearcut excitatory gastric responses were obtained in absence of a pressor response: all points localized dorsally to the pressor area. It was not possible to demonstrate any anatomic separation between points giving different types of gastric responses: they were all intermingled and moreover from the same electrode position both excitatory and inhibitory gastric responses could be elicited in different phases of the experiment depending on *ex vivo* operative procedures.

### Discussion

Gastric motility was found to be influenced by the fastigial pressor area in a most complex way. Thus in the anesthetized but otherwise intact animals either increases or decreases in gastric tone could be elicited and biphasic responses were often seen with an initial suppression followed by excitation. Concerning the excitatory gastric responses to fastigial stimulation seen in these intact animals they were at least in part mediated via the vagi and of cholinergic nature since they were eliminated or greatly reduced by vagotomy or anticholinergic agents but not by spinal cord section.

The neurogenic sympathetic inhibitory influence on gastric motility is predominantly exerted by means of inhibitory connections with the intramural cholinergic excitatory neurons (*ex vivo* Jansson and Martinson 1966). This was taken into account when the inhibitory gastric responses to fastigial stimulation were analysed. They were abolished by cholinergic

blockade or by vagotomy but reappeared if the peripheral ends of the cut vagi were stimulated simultaneously with the fastigial pressor area

After guanethidine the inhibitory responses were either abolished or reduced. In the latter case they appeared with a delay of about 20 s indicating a hormonal mechanism in all likelihood adrenal catecholamine release. It is known that guanethidine does not prevent adrenal catecholamine release whose peripheral effects are instead potentiated (Abercrombie and Davies 1963). These findings clearly indicate that the relaxations from fastigial pressor area stimulation in *intact* animals were adrenergically mediated.

*Laparotomy* profoundly changed the gastric responses to fastigial pressor area stimulation. Noxious stimuli to the abdominal cavity including laparotomy, intestinal distension *etc.* induce a marked adrenergically mediated depression of gastric motility *via* intestino-gastric inhibitory reflexes. Independent of the direction of fastigially induced gastric responses seen before laparotomy, the animal always displayed excitatory gastric responses after this procedure.

Such excitatory gastric responses might be explained either by increased activity in vagal cholinergic fibres by an inhibition of adrenergic discharge or by a suppression of prevailing activity in the vagal relaxatory fibres (Martinson 1965). Again the gastric excitatory responses were abolished by vagotomy or in some cases reduced. In the laparotomized cats it was possible to increase gastric tone by fastigial stimulation even after bilateral vagotomy provided that the distal ends of the vagal nerves were simultaneously stimulated but these gastric excitatory responses were abolished by guanethidine. This indicates that the excitatory responses seen in the laparotomized cats were due to fastigial suppression of a prevailing sympathetic influence on the stomach. This situation should be compared to that before laparotomy where fastigial stimulation led to an increased sympathetic activity to the stomach in about half the animals. Evidently laparotomy caused an activation of the intestino-gastric inhibitory reflex and this reflex is inhibited from the fastigial pressor area. Further, when transient intestino-gastric inhibitory reflexes were induced by intraabdominal injections of irritant solutions, fastigial stimulation led to a gastric motility increase by suppressing this transient reflex while the same fastigial stimulation produced motility inhibition as long as basal gastric motility was present. It is known that the intestino-intestinal inhibitory reflex can be suppressed from supraspinal levels *e.g.* from the medullary depressor area (Johansson, Jonsson and Ljung 1965, 1968). The arterial baroreceptors were on the other hand of no importance for the excitatory gastric responses induced by fastigial stimulation in laparotomized animals even though they are activated along with the fastigial pressor response.

The cerebellum has been found to influence also hypothalamically induced autonomic responses (Lisander and Martner 1971, 1973) and diencephalic lesions (Sawyer, Hilliard and Ban 1961) or precollicular decerebration (Ban *et al.* 1956) abolish some autonomic responses elicited from the cerebellum. On the other hand the fastigial pressor response persists after midbrain transection (Miura and Reis 1969, Achan and Downman 1970) while fastigially induced effects on intravesical pressure were changed (Manchanda and Bhattarai 1972). In the present study the fastigial inhibition of the intestino-gastric inhibitory reflex persisted after intercollicular decerebration indicating that the effects w

mediated via structures cranial to the midbrain. It is possible that the fastigial inhibition is mediated via the medullary depressor area which as mentioned can suppress the spinally conveyed intestino-intestinal (gastric) reflexes.

The possibility that the fastigial nucleus might increase gastric tone also by suppressing the discharge in the vagal relaxatory fibres was investigated in connection with their activation in vago-vagal reflexes. It was clearly demonstrated that this reflex could be inhibited by fastigial stimulation. This suppression was not exerted at the effector level by e.g. adrenergic or cholinergic mechanisms since the inhibition was still present after combined cholinergic (atropine) and adrenergic blockade (guanethidine or spinal cord transection). The vago-vagal relaxatory reflex is relayed in the lower brainstem since it is unaffected both by intercollicular decerebration and spinal cord transection at  $C_1-C_4$  (Jansson 1969 b). Evidence for a relay center for this reflex close to the obex has been presented by Nakazato and Ohga (1971) and the fastigial influence is likely to be exerted at this bulbar level.

Histological examination revealed that the cerebellar area from which gastric responses could be elicited was quite restricted (Fig. 6). Although the intention was primarily to investigate the fastigial pressor area, considerable parts of the fastigial nucleus and adjacent cerebellar areas were stimulated before the pressor area was identified. However, it was a regular finding that the gastric effects were almost exclusively obtained from the fastigial pressor area. There were no signs of any gross anatomical differentiation between the points causing different types of gastric responses. The technique utilized, however, does not allow for a more detailed analysis of the neuron pools involved due to current spread etc. The frequency-response curves (Fig. 5) representing the fastigially induced blood pressure rise, the gastric relaxation in intact animals and the suppression of the intestino-gastric inhibitory reflex all follow a similar course, suggesting that the neuron pools responsible for these effects operate through functionally similar mechanisms.

The results indicate that the fastigial pressor area can induce gastric relaxation by increased adrenergic sympathetic discharge and also by adrenal catecholamine release. Increased gastric tone can on the other hand be elicited via inhibition of the sympathetic intestino-gastric inhibitory reflex by increasing vagal excitatory cholinergic discharge and also by inhibition of reflexly induced activity in the vagal relaxatory fibres. Neurogenic control of the stomach is most complex and exerted at several levels. Thus, intramural neurons give rise to a basic activity which is modified by intramural ganglionic, by spinal sympathetic and by bulbar vagal reflexes. Several parts of the central nervous system, not the least hypothalamic and cortical sections, are able to influence gastric motor activity. The question arises whether these higher autonomic centres exert their effects mainly or only by modifying the activity in reflexes affecting stomach motility. In the present experiments evidence was obtained indicating that the cerebellar fastigial nucleus is able to suppress two autonomic reflexes inhibiting the stomach. Further, if gastric motor activity was high as in intact animals, the fastigial nucleus often lowered this basal (cholinergic) activity by inducing an increased sympathetic activity, perhaps by modulation of spinal sympathetic reflex arcs. In any case, the present study reveals that the cerebellum tends to increase gastric tone when it is low and decrease it when it is high. The results thus also stress the importance of the prevailing experimental conditions during explorations of central nervous control of autonomic mechanisms.

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1971 Inuchijima 1973), it is often assumed that the increased resistance is maintained almost solely by neurogenic mechanisms also in the "established" phase of SHR hypertension

Since however MAP is a product of cardiac output (CO) and total peripheral resistance (TPR) the true significance of the pressure fall subsequent to complete cardiovascular denervation can be evaluated only if both the CO and TPR components are known. In fact, perfusion experiments reveal that resistance is higher in established SHR hypertension than in NCR even during maximal vasodilatation (Folkow *et al* 1970), reflecting the presence of hemodynamically important structural changes of the SHR resistance vessels (*cf* Folkow *et al* 1973 1974) Furthermore studies on isolated perfused SHR hearts show that they display a smaller stroke volume than NCR hearts at a given diastolic filling pressure mainly due to the left ventricular hypertrophy in SHR and the consequent decrease in average myocardial diastolic stretch (Hallback, Isaksson and Norrson 1975).

Against such a background there are good reasons to believe that TPR may be elevated but CO decreased after denervation in SHR inspite of a "normalized" blood pressure compared with equally treated NCR. It was therefore decided to study more precisely the relationship between MAP CO and TPR after complete cardiovascular denervation by means of pithing in SHR and NCR.

### Methods

30 6-7 months old SHR body weight ranging between 300 to 370 g, and 30 age- sex- and weight-matched NCR were used. After anesthesia with Inactin® (5-ethyl-5 (1-methyl-propyl)-2-thio-barbituric acid Promonta), 100 mg/kg b wt., a cannula was introduced into the aortic arch via the carotid artery. The cannula was connected to a pressure transducer and a Grass polygraph for recordings of blood pressure and heart rate and subsequently used for withdrawal of arterial samples. One jugular vein was cannulated by a PE 10 catheter the tip of which was placed close to the heart. The trachea was cannulated and the animals were artificially ventilated at a rate which barely suppressed spontaneous respiration. A rhythmic inflation was used with a peak pressure of 9-10 cm H<sub>2</sub>O which was equally maintained in all animals before and after pithing. It should here be noted that the positive pressure breathing tends to reduce CO by impediment of venous return, particularly after cardiovascular denervation. Flaxedil, 1 mg/100 g was given i.p. to suppress spontaneous motility and seizures in combination with the pithing procedure. Pithing was performed by introducing a 2 mm steel rod from the atlanto-occipital joint down the entire spinal canal to destroy the spinal medulla, thereby eliminating all sympatho-adrenal activity.

CO was measured either by 1) the radioactive rubidium (<sup>86</sup>Rb) injection technique, whereby also an approximate estimation of CO distribution may be obtained (Sarnsten 1963), or by 2) the cardiogreen dye dilution technique as modified for rats.

1) CO measurement with the <sup>86</sup>Rb technique was performed on 20 SHR and 20 NCR. 10 pairs were used before and 10 pairs after the standardized pithing procedure. When pressure and heart rate were stabilized, the arterial cannula was connected to an oil-filled, constant speed withdrawal system. Exactly 20 µCi of <sup>86</sup>Rb was quickly injected by a step dispenser syringe (0.1 ml) into the jugular catheter. After the <sup>86</sup>Rb injection, carotid arterial blood was withdrawn at a constant speed of 0.67 ml/min over exactly 10 s. 30 s later the animal was suddenly killed by an i.v. injection of 0.4 ml concentrated KCl solution.

For CO determination the radioactivity in the 10 s arterial sample was counted by means of a  $\beta$ -counter. Number of counts in weighed samples of kidneys, heart, skeletal muscles and liver were used to approximate blood flow through these test organs. Even though the total <sup>86</sup>Rb amount does not enter the intracellular "sink" of the respective tissues during the first passage, there is no reason to assume that the amount of trapping in different animal groups would be different. Thus, this technique allows a fair estimation also of CO distribution in experimental animals. The <sup>86</sup>Rb method has, however the obvious disadvantage of allowing only one CO measurement per animal. Consequently measurements were first performed on one pair of SHR and NCR with intact sympathetic control, followed by another pair in which the measurements were performed during steady state condition after pithing.

2) *CO measurements with the dye dilution technique* allowed multiple determinations in each animal and therefore only 10 SHR and 10 NCR were used. At least two measurements were performed both before and after pithing. Subsequent to the two measurements after pithing most of the rats received papaverine 0.5 mg/kg in order to ensure maximal vasodilatation prior to a final CO measurement.

By means of a step dispenser syringe 0.04 ml of cardiogreen solution was injected into the jugular vein. Arterial blood (maximally 0.35 ml per recording) was withdrawn at a constant speed of 0.67 ml/min passing through a specially designed low volume (0.05 ml) densitometer cuvette. After the determination the blood was reinfused. The densitometer cuvette was connected to a Servogor recorder for recording of the dye dilution curve.

Calibration of the densitometer was performed with known concentrations of dye diluted in human plasma and the system was linear over the range of the recording scale. The accuracy of the dye dilution determinations was checked against actual plasma flows in a tube system with a mixing chamber and a pump delivering constant flows from 10 up to 90 ml/min. This check revealed a linearly increasing overestimation of the flow with the dye dilution technique as compared to the actual flow in the system, being maximally 9 per cent at flow rates of 90 ml/min. For calculating CO from the dye dilution recordings, a digitizer unit connected to a Hewlett Packard calculator was used.

## Results

Mean arterial pressure (MAP) in SHR  $198 \pm 4$  mm Hg was significantly higher than in NCR  $132 \pm 4$  mm Hg ( $p < 0.001$ ) these pressure levels being somewhat above the usual since the carotid artery cannulation implies a partial baroreceptor unloading. Table I presents mean values of cardiac output (CO), total peripheral resistance (TPR) and heart rate (HR) before and after pithing the former two parameters being expressed *per 100 g* of b wt. There was no appreciable difference in CO values obtained by the two methods and the results are therefore pooled in the Table. During the prevailing experimental conditions involving artificial positive pressure ventilation and unilateral carotid occlusion by

TABLE I Mean arterial pressure (MAP), cardiac output (CO), total peripheral resistance (TPR) and heart rate (HR) before and after pithing in SHR and NCR. Values presented are expressed as means  $\pm$  S.E. Levels of significance and per cent difference between SHR and NCR are given below. \* indicates significant difference ( $p < 0.001$ ) from control situation.

	Control conditions				After pithing			
	MAP mm Hg	CO ml/min 100 g	TPR PRU/ 100 g	HR beats/ min	MAP mm Hg	CO ml/min 100 g	TPR PRU/ 100 g	HR beats/ min
SHR n=10	$198 \pm 4$	$18.9 \pm 1.0$	$11.0 \pm 0.6$	$348 \pm 7$	$40 \pm 2$	$9.2 \pm 0.9$	$4.9 \pm 0.4$	$269 \pm 9$
NCR n=10	$132 \pm 4$	$4.0 \pm 0.9$	$5.6 \pm 0.2$	$358 \pm 9$	$44 \pm 2$	$11.1 \pm 0.9$	$3.6 \pm 0.3^*$	$61 \pm 6$
Level of sign. difference								
SHR-NCR	$p < 0.001$	$p < 0.001$	$p < 0.001$	n.s.	n.s.	$p < 0.001$	$p < 0.01$	n.s.
Per cent difference								
SHR-NCR	+51	-21	87	—	—	-35*	+36	—

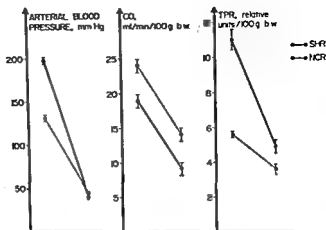


Fig. 1. Illustrates the fall in arterial pressure (left part) cardiac output (CO) (middle part) and total peripheral resistance (TPR) (right part) before and after pithing in SHR and NCR. Vertical bars indicate  $\pm$  SE.

the arterial cannulation CO was about 20% lower in SHR than in NCR in the control resting state ( $p < 0.001$ ) while TPR was nearly twice as high in SHR as in NCR ( $p < 0.001$ ).

After pithing the arterial pressures fell to about equally low levels in the NCR and SHR groups. The CO difference was even more pronounced after pithing CO being some 35 per cent lower in SHR than in NCR ( $p < 0.001$ ) the same being true for stroke volume since HR were about the same. In return TPR was now about 35 per cent higher in SHR compared with NCR ( $p < 0.01$ ). It should be noted that after pithing, eliminating neurogenic cardiovascular control and drastically lowering pressure vascular smooth muscle tone was virtually eliminated as checked by injection of papaverine which did not appreciably further reduce TPR from the postpithing values. Therefore the systemic vascular beds of NCR and SHR could be considered maximally dilated in the prevailing situation and TPR is then only dependent on the structural design of the resistance vessels and on the transmural pressures which were largely equal in NCR and SHR. — The results are also presented in a diagrammatic form in Fig. 1.

In the group of SHR and NCR studied with the cardiogreen technique the percentual fall in CO as a result of pithing could be determined in each animal. Compared with NCR a modestly though significantly greater CO reduction was observed in the SHR group ( $p < 0.05$ ) where CO was reduced by  $63 \pm 5$  per cent upon pithing while the CO reduction in NCR was  $54 \pm 2$  per cent.

Measurements of blood flow distribution in SHR and NCR by the  $^{86}\text{Rb}$  technique revealed that before pithing the cutaneous and renal blood flows were somewhat lower in SHR, while in skeletal muscle, myocardium and liver the flow was about equal in SHR and NCR. This general pattern also prevailed after pithing even though the muscle blood supply appeared to increase modestly in both groups.

In summary even after pithing and papaverine administration TPR was some 35 per cent higher in SHR than in NCR while CO and stroke volume was about 35 per cent lower in SHR. Consequently MAP reached about the same low levels in SHR and NCR when all nervous influences on the cardiovascular system were abolished.

### Discussion

The importance of the cardiovascular neurohormonal control system for the initiation and maintenance of high blood pressure in genetically hypertensive rats of the Okamoto strain (SHR) and also of the Smirk strain (GHR) has been repeatedly emphasized (e.g. Okamoto 1969 Smirk 1970 Nosaka *et al.* 1972) and many studies provide evidence of an increased neurohormonal discharge in SHR (cf. Okamoto 1969). Further also young prehypertensive SHR display an inherent evidently genetically linked hyperreactivity of central autonomic structures resulting in exaggerated increases of heart rate and arterial pressure compared with NCR whenever enhanced alertness is displayed or when the rats are exposed to "mental stress" in their daily life (Hallback and Folkow 1974). Moreover the effects of immunosympathectomy in newborn GHR and SHR (Clark 1971 Folkow *et al.* 1972) the beneficial effects of early treatment with sympatholytic drugs (Weiss 1974 Weiss Lundgren and Folkow 1974) and of longterm reduction of normal environmental stimuli (Hallback 1975) strongly suggest that central neurohormonal mechanisms are of crucial importance for the very initiation of hypertension in these animal models of human essential hypertension (cf. Folkow 1975).

Against such a background the finding that almost equally low arterial pressure levels are reached in SHR and NCR after complete cardiovascular denervation has often led to the apparently logical suggestion that the increased pressure in SHR must be essentially the result of an increased sympathetic activity also in the established phase of hypertension (Okamoto 1969 Shubayama Mirogami and Sokabe 1971). However arterial pressure depends not only on total peripheral resistance (TPR) but also on cardiac output (CO). Consequently a recording only of the product of these two parameters *i.e.* of mean arterial pressure (MAP) does not allow any conclusions concerning the mechanisms behind the pressure fall after autonomic blockade. Inuchiyama (1973) recently measured both MAP and CO before and after ganglionic blockade in 9 pairs of SHR and NCR which were younger (as an average 17 weeks old) than the present ones. As in the present study arterial pressures reached about equally low levels after denervation. TPR appeared to be higher in SHR than in NCR, although no statistical significance was obtained possibly because of the fairly small material.

The present results were obtained on a much larger experimental material and two different techniques for CO measurements were used, which did not mutually differ to any significant extent. After pithing, CO was found to be about 35 per cent lower in SHR than in NCR ( $p < 0.001$ ) and the same was true for stroke volume since heart rates were largely the same. In return TPR was largely 35 per cent higher in SHR ( $p < 0.01$ ) so that MAP was about equal in NCR and SHR. Before these results are further considered the methods used for CO measurement will be briefly evaluated. The  $^{86}\text{Rb}$  technique may be slightly influenced by the modest recirculation that occurs even though this tracer is to a marked degree "trapped" in the tissues during its first circulation. This technique will also tend to overestimate very low flow values since the standardized sampling period was relatively short (10 s) and it will further not allow for extrapolations as will the cardiogreen technique. Therefore the CO values after pithing may if anything, be slightly overestimated.

and the TPR ones correspondingly underestimated especially in SHR where CO was then particularly low

A possible disadvantage of the cardiogreen method would be the unavoidable and slightly larger blood loss during each arterial sampling (0.35 ml at most) compared with the  $^{86}\text{Rb}$  technique. However, this blood loss corresponds to only some 1.5 per cent of the total blood volume in rats of this size. Neither heart rate nor pressure revealed any significant cardiovascular influence of this small blood withdrawal which was moreover quickly re-infused to the animal. Therefore the CO methods used seem to be satisfactory for the present purpose.

A standardized positive pressure ventilation was as mentioned maintained under identical circumstances in SHR and NCR throughout the experiments. Such artificial respiration is likely to induce cardiovascular reflexes by the impediment with venous return which tends to reduce enddiastolic filling pressure. Such interferences not present during normal conditions might to a different extent influence CO in NCR and SHR. Thus control CO was about 20 per cent lower in SHR than in NCR in the prevailing situation while no such difference is found when anesthetized SHR and NCR are compared without artificial respiration (own preliminary results).

After spinal destruction eliminating all cardiovascular reflex adjustments the systemic vessels became virtually maximally dilated as checked by papaverine. TPR is in this situation essentially determined by the structural design of the resistance vessels and by their prevailing transmural pressure which was largely equal in NCR and SHR. Therefore the 35 per cent higher TPR after pithing compared with NCR ( $p < 0.01$ ) indicates a structural narrowing of the SHR resistance vessels in agreement with previous findings in artificially perfused maximally dilated systemic vascular bed in SHR and NCR (Folkow *et al.* 1970). Further hemodynamic analyses indicate an increase in wall/lumen ratio confined to the precapillary resistance vessels and mainly due to a rapidly established media thickening (Folkow *et al.* 1973, 1974). Due to this precapillary structural autoregulation in SHR both excitatory and inhibitory influences result in exaggerated precapillary luminal changes for given changes in smooth muscle activity. An important positive feedback interaction is thus created between functional excitatory and structural influences so that a higher resistance may be maintained in SHR by the same neurogenic activity as in NCR (*cf.* Folkow 1975). However, it also implies exaggerated resistance and pressure reductions in SHR compared with NCR when as in this particular case all neurohormonal excitatory influences are suddenly eliminated (Fig. 1).

Further the present results show a relatively more pronounced CO reduction in SHR than in NCR upon complete denervation. In this situation cardiac performance is mainly determined by the myocardial heterometric and homeometric autoregulation (*cf.* Sarnoff and Mitchell 1962). Since MAP was largely equal in NCR and SHR after pithing the diastolic filling pressure will primarily determine CO since inherent heart rates proved to be largely equal in NCR and SHR in this case. Thus stroke volume was about 35 per cent lower in SHR than in NCR after pithing which may in part be explained by a relatively more pronounced interference with the SHR myocardial blood supply as a result of the marked fall in pressure head. More important is probably the fact that SHR left ventricles

a lower stroke volume for a given enddiastolic pressure compared with NCR. The reason is that the average myocardial stretch becomes reduced largely in proportion to the left ventricular hypertrophy shifting the Frank-Starling curve for SHR to the right in the physiological range of filling pressures (Hallback, Isaksson and Norell 1975). Consequently the sympathetic control of the low pressure capacitance compartments might become increasingly important along with the development of SHR hypertension, being here needed to ensure a proper filling of the gradually less compliant left ventricle.

In summary, the hemodynamic pattern in SHR and NCR after complete cardiovascular denervation in the prevailing situation associated with maximal systemic vasodilatation is in SHR characterized by a 35 per cent higher TPR and a proportionally lower CO and stroke volume compared with equally treated NCR. These opposite changes in TPR and CO after denervation lead to about equal MAP levels in SHR and NCR. Both these changes are consequences mainly of the rapidly established structural adaptation of the cardiovascular high pressure compartments occurring in hypertension and involving not only the left ventricle and systemic arteries but also the precapillary resistance vessels.

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## Rapid Transport of Noradrenaline in Adrenergic Axons of Rat Sciatic Nerve Distal to a Crush<sup>1</sup>

By

J HÄGGENDAL, A DAHLSTRÖM and P A LARSSON

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### Abstract

HÄGGENDAL, J A DAHLSTRÖM and P A LARSSON *Rapid transport of noradrenaline in adrenergic axons of rat sciatic nerve distal to a crush*. Acta physiol scand 1975 94 386-392

The transport of NA in the rat sciatic nerve distal to a crush was studied in 5 mm segments. The disappearance of a NA fraction in consecutive segments with time after crushing (0-12 h) was interpreted to indicate a proximo-distal migration of a NA fraction into further distal parts of the nerve. This transportable NA fraction was found to be about 45% of the NA in normal sciatic nerves and migrated at a rate of approximately 8 mm/h. The apparently non mobile fraction (55%) was probably mainly located in vaso-constrictor nerve terminals of the blood vessels supplying the nerve. This non mobile NA fraction does not contribute to the NA accumulations proximal to a crush. Thus, when calculating the rate of transport of any substance from accumulation experiments, corrections for a non mobile fraction must always be considered.

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In adrenergic neurons noradrenaline (NA) storage granules are transported intra axonally towards the nerve terminals (cf Dahlström and Häggendal 1966, Banks, Mangnall and Mayor 1969). Following a nerve crush NA rapidly accumulates in the adrenergic axons on the proximal side of the crush. Most of the accumulated NA is probably stored in amine storage particles as shown by the reserpine-depletability (Dahlström 1963, 1967 a). Furthermore, the synthesis and break-down of NA in the nerve segment just above the crush are balanced (McLean and Keen 1972). Therefore, the time-course curve for NA accumulation has been used to calculate the rate of transport of amine storage granules. Thus, the NA has been used as a marker for the storage particles. Using this procedure, the rate of transport in rat sciatic nerve axons was found to be about 5 mm/h (Dahlström and Häggendal 1966). The calculations were, however, based on the assumptions that a) all the NA in the sciatic nerve was transportable and that b) the amine granules stored a constant amount of NA during their transport and accumulation.

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<sup>1</sup> Reported in part at the Wenner-Gren Symposium on "Dynamics of Degeneration and Growth in Neurons" Stockholm, May 1973.



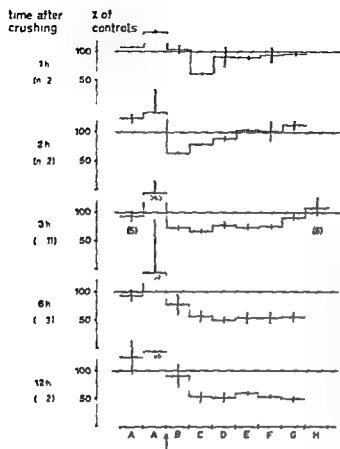


Fig. 1 The NA content in 5 mm segments of the rat sciatic nerve I-12 II after crushing. Segments A<sup>1</sup> and A proximal to the crush, segments B-H distal to the crush (arrow). Means  $\pm$  S.E. are given in of the normal values of the respective segment (see Table I). There is no quantitative evidence for a retrograde transport in segment II 2 II post crushing. The gradual rise later could be caused by a small retrograde accumulation but may also be due to contamination from segment A at the dissection.

where the results are expressed in per cent of the values of the corresponding control segment. As shown previously the NA content in segment A just above the crush increased rapidly with time (Fig. 1).

In the part distal to the crush (Fig. 1) a clearcut decrease could be seen already after 1 II in segment C. After 2 h the decrease involved segments B and C with a tendency to some decrease also in segment D. Three h after crushing the NA decrease was seen in all distal segments except the 2 most distal ones (G and H) involving 5 segments distal to the crush. In the 6 segment (G) there was in one series of experiments (3 h, I) a clearcut decrease, while in another series (3 h, II) no decrease was found. All segments showed a decreased NA content 6 and 12 II after crushing, to about 55% of normal (C-G). In segment II the NA content was normal at 1 II low at 2 h and gradually rising between 2 h and 12 h after crushing (Fig. 1).

Since it was of interest to study where the remaining 55% of NA was located the connective tissue sheath and the nerve fibre core were assayed separately. The results are shown in Table II. The 2 cm nerve part assayed corresponds to the segments C-F in Fig. 1. Part B was omitted because of the possibility that retrograde accumulations (cf. Dahlström 1965) could interfere with the results. About 22% (0.5 ng) of the NA within a normal 2 cm

TABLE II The NA content (ng per 2 cm of nerve) in the connective tissue sheath and in the core of axon bundles respectively of rat sciatic nerve. The dissections were made on an ice-cold glass plate as indicated in the text. Uncrushed nerves (normal) and nerve segments distal to a 6 h crush were used. n.s. indicates not significantly different from normal values

Experiment no	Normal		6 h crush		Decrease in % of normal	
	Sheath	Core	Sheath	Core	Sheath	Core
1	0.48	1.66	0.46	1.14	4	31
2	0.45	1.72	0.33	1.14	27	34
3	0.58	1.88	0.39	1.02	33	46
Mean value	0.50 ± 0.04	1.75 ± 0.07	0.39 ± 0.04 (n.s.)	1.10 ± 0.04 (p < 0.005)	21	37
Total (sheath + core)	2.25 ± 0.10		1.49 ± 0.06 (p < 0.005)		35 ± 5	

segment was present in the connective tissue sheath. Distal to a 6 h crush about 35% (0.4 ng) of the total NA content (sheath + core) was found in the sheath. The content of the core (axons and some connective tissue) was significantly decreased by 37% 6 h after crushing. A small decrease was also noticed in the sheath but this decrease was not statistically significant.

The histochemical studies revealed the presence of a large number of adrenergic NA containing nerve terminals in blood vessels of the connective tissue sheath (Fig. 2). A few non terminal axons were also observed. In the core a few vessels with adrenergic nerve terminals were observed situated in the thin connective tissue of the endo- and perineurium between the myelinated and non myelinated axons.

Since it appeared impossible to totally separate nerve terminals in the connective tissue from axons in the sciatic nerve the quantitative studies in Table II were not extended.

### Discussion

The results of the present study (Fig. 1) demonstrate a clearance of NA progressing distally in the nerve below the crush with time after crushing. This decrease of 45% is most probably caused by a further transport distally of this NA fraction. The distal transport occurred in spite of the interrupted connection with the cell bodies in agreement with earlier reports on a redistribution of NA in double ligated sciatic nerves (Dahlstrom 1967 b). The fast transport of amine granules distal to a crush is also in agreement with studies on the intra axonal transport of labelled proteins distal to a crush (Ochs and Ransjö 1969).

Some information about the rate of the fast transport distal to the crush can be obtained from the disappearance pattern. 1 h after crushing the NA decrease was observed at least within one 5 mm section of the nerve (segment C, Fig. 1). The rate of transport therefore appears to be at least 5 mm/h.

2 h after crushing the NA clearance was observed within at least 2 segments (B + C) with a tendency to decrease also in segment D. The distance may thus be more than 10 mm and



Fig. 2 Fluorescence micrograph of an air dried stretch preparation of the connective tissue sheath of a normal rat sciatic nerve. Small arteries with strongly fluorescent adrenergic nerve terminals can be observed ( $\rightarrow$ ) ( $\times 160$ ).

the time for active transport was about 2 h. The rate of distal transport thus appeared to be between 5 and 10 mm/h.

3 h after crushing, the NA was decreased within a distance of at least 25 mm from the crush (segments B 1). The time for active transport was about 3 h which will give a transport rate of about 8 mm/h.

6 and 12 h after crushing, the decrease in NA was observed in all investigated segments indicating that the transportable NA had been transported into segments further distally than the most distal segments observed in the nerve. The distance for transport is thus longer than 30 mm and the rate probably faster than 5 mm/h.

These results indicate that the rate of NA transport distal to a crush is faster than 5 mm/h but lower than 10 mm/h and probably around 8 mm/h. Shorter nerve segments would have to be used to obtain more exact values. This would be difficult for practical reasons since then more than 20 nerve parts would have to be pooled with the present method.

The maximal decrease of NA in the nerve segments distal to the crush was found to be 45% of the normal content (Fig. 1). This was obviously the fraction of NA which had been transported further down the axons after the crush indicating that in the rat sciatic

nerve only 45% of the NA content is transportable. The remaining 55% appears to be either stationary or very slowly migrating.

The location of the stationary or slowly migrating fraction of NA was studied. In the connective tissue sheath assayed separately for NA content (Table II) a large amount of NA was present. Histochemical studies of air-dried spread preparations of the sheath revealed the presence of many blood vessels with NA containing nerve terminals (Fig. 2). Also in the core preparation consisting mainly of different kinds of axons some adrenergically innervated blood vessels were seen in the endo- and perineural connective tissue. It is therefore likely that at least most of the stationary NA is located within adrenergic vasomotor nerve terminals. It cannot be excluded that a small part of the stationary NA may be located inside the adrenergic non terminal axons in a form which does not permit rapid intra axonal transport.

In the sheath preparation (Table II) a small non-significant decrease of about 20% was observed. Since non terminal axons were observed histochemically in the sheath preparation a distally directed transport in these axons may explain the decrease in NA. Likewise a NA decrease of 45% would be expected to occur in the core preparation 6 h after crushing if this preparation contained all the adrenergic axons and no nerve terminals. A decrease of only 37% was however observed which may be due to the presence of adrenergic nerve terminals (not losing NA after crushing) in vessels around the axon bundles. The total decrease in the dissected 2 cm segments after crushing (sheath and core taken together) was 35% i.e. 10% less than what would be expected from the data in Fig. 1. This may be due to losses of axonal NA from the core during the preparation procedure.

In cholinergic nerves a rapid transport of acetylcholine (ACh) has been demonstrated (Häggendal *et al* 1971, 1973; Dahlström *et al* 1974). In these nerves (sciatic motor nerve and autonomic preganglionic nerve) the fraction of ACh which is rapidly transportable distal to a crush is only about 20 and 5% of normal respectively. In these nerves the stationary fraction is probably not located in vasomotor nerve terminals since the connective tissue sheath (carrying most of the blood vessels) contained no detectable amounts of ACh (Dahlström *et al* 1974).

The fact that only a part of the NA content in the sciatic nerve is transportable is of great importance for calculations on the rate of NA transport using values for the NA accumulation proximal to a crush. Earlier a rate of 5 mm/h was calculated based on the assumption that all of the NA was transportable (Dahlström and Häggendal 1966). With the present information that only 45% is mobile and using the new information on the NA loading of the granules (see accompanying paper by Dahlström *et al* 1975) a rate of 9 mm/h has now been calculated which is in good agreement with the figure of 8 mm/h for transport distal to a crush obtained in this study. This clearly demonstrates the importance of estimating the transportable fraction of any substance investigated for intra axonal transport by accumulations proximal to a crush or ligation (*cf* Häggendal *et al* 1973; Dahlström 1973).

Dopamine  $\beta$ -hydroxylase (D $\beta$ H) an enzyme bound to the amine storage granules has been studied with respect to intra axonal transport and rapidly transportable fraction. About 30% of the normal D $\beta$ H content in the sciatic nerve (Wooten and Coyle 1973) and

less than 50% (Häggendal *et al* 1974) was found to be rapidly mobile. Based on these values and on accumulation data above a nerve crush a rate of transport of 6–8 mm/h was calculated for DβH (Wooten and Coyle 1973). This is in excellent agreement with the present data for transport of NA storage granules using NA as a marker instead of DβH.

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## Synaptosomes Containing Large Agranular Vesicles Isolated from Developing Rat Brain

By

L. KANERVA\* A. HERVONEN\* \* and A. H. TISSARI\*\*

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### Abstract

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KANERVA L. A. HERVONEN and A. H. TISSARI *Synaptosomes containing large agranular vesicles isolated from developing rat brain* Acta physiol scand 1975 94 393-397

Using the subcellular fractionation technique the fine structure of the isolated nerve endings (synaptosomes) from the hemispheres and brain stem of the 1-day old and adult rat was examined. In the synaptosomal fractions of the brain of 1-day old rats we observed a new type of nerve endings containing predominantly large agranular vesicles about 1 000 Å in diameter. After incubation with  $\alpha$ -methylnoradrenaline or 5-hydroxydopamine these vesicles remained agranular. It is assumed that the new type of large vesicles represent developing synaptic vesicles.

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The subcellular fractionation technique (De Robertis *et al* 1961 Gray and Whittaker 1962) gives a unique possibility of studying large numbers of nerve endings with electron microscopy and of observing morphologically different types of nerve endings within a reasonable number of tissue sections. This technique seems especially suitable for studies of immature nervous tissue which has a low frequency of nerve terminals at different degrees of maturation (for ref. see Hervonen *et al* 1974 Kanerva *et al* 1974 a). We have used the subfractionation technique to study by electron microscopic and biochemical means maturation of nerve ending during ontogeny in rat and human brain (Hervonen *et al* 1974 Kanerva *et al* 1974 b Tissan 1973 1975). Information on the ultrastructure of synaptosomes from developing brain could also contribute to an understanding of the origin and significance of different types of synaptic vesicles and other intraneuronal structures. We have observed in the brain of 1-day old rats a new type of synaptosomes containing predominantly large agranular vesicles described in the present paper.

### Material and Methods

Adult and 1-day-old Sprague-Dawley rats were used. In some experiments animals pretreated with a monoamine oxidase inhibitor pargyline HCl (Eutonyl® 100 mg/kg i.p. 1 h before killing) were included. Subfractions were prepared from the hemispheres and brain stem as previously described (see Hervonen



*et al.* 1974) The crude mitochondrial fraction ( $P_2$ ) was resuspended in 0.32 M sucrose and layered over a discontinuous sucrose gradient consisting of layers of 1.4, 1.0 and 0.8 M sucrose. The gradient was centrifuged and the layers were collected by aspiration, diluted with water to sucrose concentration 0.32 M for A and B and to 0.55 M for C, D and E. Subfractions were centrifuged and the pellets suspended in Krebs bicarbonate solution pH 7.4 (KRB). In some experiments fraction  $P_2$  prepared from the striatum and hypothalamus of the adults and from the hemispheres, diencephalon, mesencephalon and pons medulla oblongata of the 1 day old rats was used and suspended directly in KRB. Aliquots were removed at 0°C, suspensions preincubated at 37°C and 95%  $O_2$ -5%  $CO_2$  in a water bath shaker for 8 min and L- $\alpha$  methyl noradrenaline HCl ( $\alpha$  methyl NA, Corbasil<sup>®</sup>) or 5 hydroxydopamine HCl (5 OH-DA, H 83/35) added to final concentrations of 10 or 100  $\mu$ g/ml. Ascorbic acid to 0.2 mg/ml was also added to some samples. Incubation was continued for a further 15 and 30 min, aliquots were removed and cooled in an ice bath. Controls without substrate were incubated for the same period. Samples were fixed either as a suspension or pellet in  $KMnO_4$  (final concentration 3% in 0.1 M sodium phosphate buffer pH 7.0 for 30 to 60 min) or 2.5% glutaraldehyde (in 0.1 M sodium phosphate buffer pH 7.4 for 30 to 180 min). Pellets were sedimented by centrifugation at 10 000  $g$  for 15 min and washed after fixation in the buffer for 1 h. The glutaraldehyde fixed specimens were postfixed in phosphate buffered 1%  $OsO_4$  at pH 7.4 for 1 to 2 h. Then the specimens were dehydrated and embedded in Epon Araldite. The  $KMnO_4$  fixed sections were viewed and photographed unstained while the glutaraldehyde fixed specimens were stained on grids with lead citrate and uranyl acetate. A Philips EM 300 electron microscope operated at 40 to 60 kV was used.

### Results and Discussion

Most of the synaptosomes of the adult and 1 day-old rats contained mainly small agranular spherical vesicles and a few large granular and agranular vesicles after fixation in glutaraldehyde followed by osmium tetroxide or in  $KMnO_4$ . After incubation with  $\alpha$  methyl NA or 5 OH-DA and fixation in  $KMnO_4$ , small granular vesicles were found in less than 1% of the synaptosomes of the fraction  $P_2$  or subfractions of the adult and even less frequently in those of the 1-day-old rats. A third type were the synaptosomes which contained predominantly large agranular vesicles about 1 000 Å in diameter and no or a few small agranular vesicles after  $KMnO_4$  fixation and were found in the brain of 1 day-old rats (Fig. 1). These synaptosomes occurred in all subfractions which contain nerve endings in 1 day-old rats namely A, B, C and D. After incubation with  $\alpha$  methyl NA or 5 OH-DA these vesicles remained agranular. After  $KMnO_4$  fixation the internal structure of the large agranular vesicles consisted of fine reticulum the density of which varied only slightly from vesicle to vesicle and was slightly higher than that of the surrounding axoplasm. Some of these vesicles appeared elongated (Fig. 1). The synaptosomes with the large agranular vesicles also contained the normal organelles of nerve endings, small mitochondria, occasional neurotubuli and probably a greater abundance of smooth endoplasmic reticulum than the other types of synaptosomes. In adult rats synaptosomes containing an abundance of large agranular vesicles could be detected less often.

In the peripheral nervous system axons containing large opaque or agranular vesicles have been demonstrated and attributed to non-cholinergic (purinergic) inhibitory nerves (Robinson *et al.* 1971; Burnstock 1972; Hervonen 1973) while the inhibitory nerves of the central nervous system have been connected with flattened small agranular vesicles (Uchizono 1965).

In studies of the ultrastructure of developing adrenergic innervation of rat pineal gland Machado (1971) found that the proportion of small granular vesicles increases with age and that of small agranular vesicles decreases while the absolute number of large granular vesicles



Fig. 1 Synaptosomes of crude mitochondrial fraction (P<sub>1</sub>) prepared from the hemisphere of one-day-old rat (the animals were pretreated with pargyline and the fraction was incubated with 10  $\mu$ g/ml 5-OH DA for 30 min). One synaptosome (a) contains a moderate number of small agranular synaptic vesicles, a few large agranular vesicles (thin arrow) and a mitochondrion (m). The other nerve ending (b) contains many large agranular vesicles, some of which appear flattened (thick arrow). The agranular vesicles have an internal structure consisting of fine reticulum. The nerve ending also contains many arrays of endoplasmic reticulum (asterisk). Fixed on as a suspension in 3% KMnO<sub>4</sub> 66 000.

decreases slightly. He concluded that large granular vesicles might have some functional significance in the early development of adrenergic fiber but might not be young forms of small granular vesicles as suggested by Gelfen and Ostberg (1969). Vesicles may be formed from smooth endoplasmic reticulum (SER) and the dense material may appear in the SER before and after (large granular vesicles) or after (small granular vesicles) the vesicles have pinched off from the reticulum (Machado 1971). Almost all components of the neuron have been proposed as the origin of the synaptic vesicles: mitochondria, nerve cell membrane, complex vesicles, large granular vesicles, the Golgi apparatus and the smooth endoplasmic reticulum (for ref. see Kanerva *et al.* 1974a). The endoplasmic reticulum has also been suggested as a site of amine store (Tranzer and Thoenen 1967; Eranko 1972; Tranzer 1973). In ligated rat sciatic nerve proximal to the ligation, local morphogenesis involves rapid outgrowth of endoplasmic reticulum and appearance of empty vesicular and tubular profiles (Taxi and Sotelo 1973). In the present study synaptosomes of the 1-day-old rat containing large agranular vesicles had also smooth endoplasmic reticulum abundantly and elongated vesicles, possibly newly formed ones. Interestingly, in a phylogenetically old group, the cephalopods, large agranular vesicles have been found in the synaptosomes of the adult octopus vulgaris (Jones 1967).

It is assumed that the new type of large vesicles described in the present paper represent non-degenerative developing synaptic vesicles. Their transmitter nature is not known although the possibility of monoaminergic origin does come to mind. Further study on ultrastructure of developing synaptosomes is in progress. A grant from the Paulo Foundation is gratefully acknowledged.

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## Group II Excitation in Motoneurons and Double Sensory Innervation of Extensor Digitorum Brevis

By

A LUNDBERG, K MALMGREN and E D SCHOMBURG<sup>1</sup>

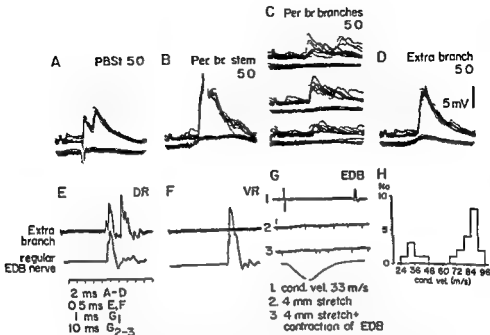
In a recent investigation (Lundberg *et al* 1975) of the effects of electrical stimulation of group II muscle afferents we noted that very large EPSPs are evoked in flexor motoneurons from the nerve to SPM (superficial peroneal muscles: peroneus longus, tertius and brevis). By contrast the effect from the nerve to DP (deep peroneal: tibialis anterior and extensor digitorum brevis) was weak or entirely lacking. The explanation may be either that secondary spindle afferents from the two groups of muscles differ with respect to their central actions or else that the effect from the SPM nerve is evoked from afferents of non-spindle origin.

As part of a further analysis we have now investigated separately the effect from the nerves to peroneus longus, tertius and brevis. Intracellular recording was made from knee flexor motoneurons. It was a regular finding that group II volleys from peroneus tertius or longus evoked only small EPSPs and that virtually the entire SPM group II effect was produced from the peroneus brevis nerve. However, we noted that this nerve contains a small *extra branch* which does not go to peroneus brevis but continues along the dorsal surface of the peroneus brevis tendon to enter the foot on the dorsal side of the lateral malleolus. Fig 1 B-D shows that a major part of the group II EPSP from the peroneus brevis nerve is not evoked from the proper branches to this muscle (C) but from the extra branch to the foot (D). Stimulation of this branch did not evoke any muscular contraction in the foot and we expected that its fibres originated entirely from extramuscular tissue. However, dissection of the course of this branch in the foot (5 cats) revealed that it passes into extensor digitorum brevis (EDB) and pulling the transected tendon gave a multifibre discharge which had all the characteristics of a discharge in muscle spindle afferents.

EDB is known to be innervated via a nerve which runs with the DP nerve and courses distally along the inner surface of tibialis anterior. On reaching the dorsum of the foot it divides into a lateral branch to skin and a medial branch which passes into EDB. We have dissected the latter branch and compared its electroneurogram evoked by stimulation of the appropriate ventral and dorsal roots with that recorded in the extra branch to EDB.

Fig 1 E, F show that all  $\alpha$ - and  $\gamma$ -afferents to EDB are in the regular nerve. Both branches contain group I and II afferents but the former predominate in the regular nerve and the

<sup>1</sup> Present address: Institute of Physiology, University of Göttingen, F.R.G.



**Fig 1** A-D upper traces intracellular recordings from a motoneuron to posterior biceps-semi-tendinosus (PBSt) lower traces from the dorsal root entry zone in L7 A 1a and group II EPSP evoked from the PBSt nerve B-D EPSPs from Per br stem and branches from it as indicated see text and observe also similarity in negative cord dorsum potential in B and D E-F recording from extra branch (dist. 23.4 cm) and regular nerve to EDB (dist. 25.9 cm) on stimulation of appropriate dorsal (DR) and ventral root (VR) as indicated Strength of stimulation in A-F 50 times threshold G 1-3 single unit dorsal root filament recording from secondary EDB afferents running in the extra branch which was in intact connexion with EDB the EDB tendons were dissected free and connected to strain gauge and pulling device Contraction of EDB by stimulation of efferents in regular nerve H histogram of conduction velocities of spindle EDB afferents in the extra branch. See text. Spinal cats anesthetized with chloralose

latter in the extra branch. The fastest group I afferents in the extra branch conduct at 90 m/s vs 105 in the regular nerve. Single unit recordings from dorsal root filaments confirmed that spindle afferents from EDB primaries as well as secondaries course in this extra branch. The graph in H gives the distribution of conduction velocities in spindle afferents found in a systematic analysis of all dorsal root filaments with axons in the extra branch (regular nerve cut). The arbitrary setting of 72 m/s as dividing line (cf Matthews 1972) gives 16 primary and 7 secondary afferents in the extra branch. However the distribution in two separate groups in H rather suggests a general downwards displacement of conduction velocities. If so all the fibres between 96-66 m/s may be primaries although a conduction velocity above 80 m/s usually is required in order to attribute with some confidence a spindle afferent to a primary ending (Matthews 1972). EDB has 56 muscle spindles (R and Davey 1968) and if we assume that some secondaries were missed in the filament analysis then we arrive at the suggestion that the extra branch contains about 25 of the spindle afferents from EDB. They seem to originate mainly from the lateral head of EDB.

the extra branch enters. Spindle afferents from the two medial heads run in the regular nerve as evidenced by the mass discharge evoked by separate pulling of their tendons. In summary our results show that there is a double nerve supply to EDB:  $\alpha$  and  $\gamma$ -efferents are entirely in the regular nerve to EDB while part of the spindle afferents run in a separate nerve the extra branch which joins the peroneus brevis nerve.

The problem at hand is whether the large group II EPSP evoked in knee flexor motoneurons on electrical stimulation of the extra branch (D Fig. 1) is evoked from secondary EDB afferents or from non-spindle afferents. The dorsal root filament analysis revealed that the prominent group II peak in the extra branch (Fig. 1 E) is contributed largely by non-spindle afferents. We found 61 group II fibres from receptors outside EDB, most of which were activated by strong deep pressure of the toes or the metatarsi but some from the skin of the lateral aspect of the foot. Thus the ratio of secondary afferents to non-spindle group II afferents in the extra branch is about 1:10. The synaptic activity evoked via the extra branch on adequate stimulation of the foot was investigated with intracellular recording from knee flexor motoneurons. Stretch of EDB gave only very weak excitatory synaptic noise, while marked activity was produced by pressure of the toes or metatarsi. Altogether the present findings strongly suggest that the large group II EPSP evoked in knee flexor motoneurons from the extra branch is mainly due to activation of afferents from extra muscular receptors in the foot.

This work was supported by the Swedish Medical Research Council (Project No. 94).

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## The Capillary Permeability of the *rete mirabile* of the Eel, *Anguilla vulgaris* L

By

ØVERRE STRAY PEDERSEN and JOHAN B STEEN

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### Abstract

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Direct measurements of transcapillary exchange during steady-state conditions have been performed in the *rete mirabile* of the eel. The capillaries were found to be more than 30 times as permeable to THO ( $P_{\text{THO}} = 33.2 \times 10^{-5} \text{ cm sec}^{-1}$ ) ethanol and antipyrine than to  $\text{K}^+$ . The mutual relationship of the permeabilities for  $\text{K}^+$ ,  $\text{Na}^+$ , urea and sucrose were similar to that between the corresponding free diffusion coefficients in water. The permeability characteristics did not change when the perfusate contained metabolic inhibitors. Addition of 1 mM/l of albumin lead to a significant transcapillary osmotic flow.  $\text{NaCl}$ , urea and sucrose however did not cause osmotic flow. Based on the quantitative values for permeability and volume flow the capillary pores should be about 1300 Å and have a frequency of  $1.3 \times 10^5$  per  $\text{cm}^2$  of the capillary surface occupying an area of about 0.1% of the total area. The results obtained in the present investigation raise questions as to the validity of the Pappenheimer single pore theory for transcapillary transport as well as to the fine structure of the capillary pores. As an alternative to Pappenheimer's theory a multi-pathway model is proposed in order to explain transcapillary transport.

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The capillary wall is made up of flattened endothelial cells and a basement membrane. The width of the cell junctions (pore or slit diameter) varies considerably from one organ to another. In skeletal muscles of mammals it is about 40 Å (Karnovsky 1966). In the brain capillaries the outer leaflets of the apposed cell membranes appear to be fused (tight junctions) (Reese and Karnovsky 1967).

There is general agreement that the transport of gases, water, ions and small substances across the capillary barrier is a passive diffusion process (Kruhoffer 1946). Lipid-soluble substances diffuse mainly through the endothelial cells, whereas water-soluble substances pass through the capillary wall via the intercellular clefts.

Pappenheimer *et al.* (1951) and Pappenheimer (1953) presented an extensive and detailed model for the porous exchange. They based their model on the results obtained from studies of the osmotic transients of different substances during perfusion experiments on cat hind limbs. They found that the osmotic effect of molecules of varying sizes could be quantita-



tively accounted for by assuming that 1) transport of both water and other substances occurred only through the pores and 2) the pores restricted diffusion progressively the closer the diameter of the substance was to that of the pore.

Later research has lent both support and doubts as to the validity of this model. Beck and Schultz (1972) established that mica sheets with uniform straight pores with diameter of 90 Å cause approximately the same molecular size dependant restriction to diffusion as postulated for capillaries by Pappenheimer *et al* (1951). Michel (1970) has shown that the permeability to Patent Blue and ions of the mesenteric capillaries of the frog is far greater at the clefts than across the cells. But it has also been shown in the rabbit placenta (Faber and Hart 1967) and in the dog heart (Alvarez and Yudilevich 1967) that the capillaries are significantly more permeable to THO than should be expected if the slits were the only pathway for diffusion. Crone (1963) was not able to demonstrate any diffusional restriction to sucrose and inulin in muscle capillaries whereas a considerable restriction to Cr EDTA and inulin was found by Trap-Jensen and Lassen (1971). In the capillaries of the dog heart Alvarez and Yudilevich (1969) found no restriction to smaller molecules whereas Schafer and Johnson (1964) and Vargas and Johnson (1967) found permeability values for sucrose and inulin strongly indicating a restricted diffusion.

Capillaries are unfortunately not readily accessible for permeability studies. In the investigations mentioned different methods have been employed, such as the indicator dilution method and other modifications of clearance procedures and the osmotic transient method. All of these techniques give indirect data and can never be carried out under steady state conditions. Also, since the capillaries make up only a minor fraction of the tissue, it might be questioned whether the permeabilities obtained from these studies actually reflect the properties of the capillary membrane proper or involve diffusional properties of extravascular tissue and fluid as well.

The capillary bundle of the *rete mirabile* connected to the swimbladder of the eel appeared to us as an organ of choice for studies of capillary exchange. Krogh (1929) called attention to the unique anatomy of the *rete*. A single *rete* has a cross-sectional area of 8 mm<sup>2</sup> and contains 58 000 arterial and 44 000 venous capillaries. The capillary surface area in each of the capillary systems is as great as 53 cm<sup>2</sup>. These measurements have been confirmed by Stray Pedersen and Nicolaysen (1975).

Electron microscopical studies of the capillaries have been performed by Dorn (1961) and Fawcett (1961) and by Stray Pedersen and Nicolaysen (1975). The arterial capillaries have endothelial cells of variable thicknesses from 0.2–1 µm. The average width of the junctions between the cells is 110 Å. The endothelial cells of the venous capillaries are thinner, about 0.15 µm and richly fenestrated. The junctions are somewhat wider (average width 120 Å). Both types of the endothelial cells contained numerous (pinocytotic) micro-vesicles and several giant vacuoles. The arterial capillaries may therefore be compared to the capillaries of skeletal and heart muscles of mammals whereas the venous ones are morphologically similar to those found in the intestinal mucosa of mammals.

Perfusion of the *rete* with fluids of known compositions allowed direct measurements of the steady-state exchange of substances as well as of osmotic and hydrostatic flow between the arterial and venous capillaries. On the basis of these experiments we were able to postulate

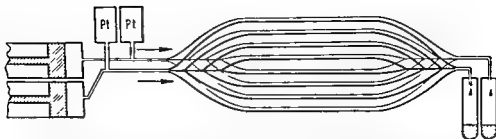


Fig. 1 Schematic drawing of the experimental design (parallel perfusion). The perfusates, one reference solution containing the test substances and one zero-solution, were delivered to the *rete* from two syringes (left in figure) in a pump. The input pressures were measured by pressure transducers (Pt) connected to the input cannulas by T tubes. The outputs from the *rete* were collected in two tubes (right in figure). The outflow was measured by weighing the tubes before and after the sampling. The concentrations of the substances to be studied were measured in the output samples as well as in the input solutions.

a physiological model of the capillary membrane in the *rete* and make a comparison between this and the capillary model generally accepted.

## Methods

### Operation technique

The eel was anesthetized by submersion in a 0.05% solution of MS-222 in water or made unconscious by a blow on the head. The blood supply to the swimbladder was ligated and the organ removed. The bladder was opened, spread out and fastened by pins. Polyethylene cannulas were inserted in the artery and vein on the proximal (heart) side of the *rete* (see Fig. 1) and the organ was flushed with heparinized plasma containing papaverine in order to wash out the blood cells. Small glass tubes were inserted in an artery/vein pair on the bladder side of the *rete* and fastened by ophthalmic suturing materials. The numerous side branches from these main vessels were closed by special broad-plated bulldog clamps.

The input tubes of the *rete* were connected to a perfusion pump and output fluid was collected in test tubes placed below the preparation plate (Fig. 1).

### Perfusion

Constant flow perfusion of the *rete* was carried out with Unita Perfusion Pumps (Braun-Melsungen) with an accuracy within 0.2% of the delivery.

The perfusion pressure in both input cannulas was continuously recorded by connecting a side branch to a Statham P23De transducer (Fig. 1). This served as a continuous control of the perfusion conditions. The main type of accidents occurring in these micro-perfusions was that of tiny air emboli in the perfusates. If these were not eliminated immediately, the experiment was spoiled.

The flow rate was normally adjusted to give a perfusion pressure of about 30–40 cm H<sub>2</sub>O in the input artery. This pressure corresponds to the mean arterial pressure measured in the dorsal aorta of eels (Mott 1954) and to the blood pressure in the swimbladder artery (own measurements). At such conditions the perfusion pressure in the venous input most often was in the range of 10–15 cm H<sub>2</sub>O.

In most experiments the arterial and venous system of the *rete* was perfused in a parallel fashion using the vessels at the heart pole of the *rete* as input vessels. During parallel perfusion the risk for shunts and leakages to occur was less than with counter-current flow. In some experiments, however, counter-current perfusion was employed. Samples of output were collected for 2–15 min depending on the flow rate. The samples were weighed and the outflow rate determined.

### Perfusates

Horse plasma was obtained by centrifugation of heparinized horse blood (25 IU/ml of Heparin A.L.) for 15 min at about 1000 g. The plasma was divided in appropriate batches and frozen. Prior to an experiment a plasma batch was thawed at room temperature and filtered through one layer of filter paper.

TABLE I Relevant physico-chemical data of the test substances

Substance	MW	Molecular (ionic) radius (Å)	$D_s$ ( $\times 10^5$ cm sec $^{-1}$ )	Olive oil/water partition coefficients
(Water)	18	1.5	2.66 [1]	0.007 [6]
THO	20	1.5	2.44 [1]	do (?)
Na <sup>+</sup>	23	0.95 (as nonhydrated)	1.48 as NaCl [7]	Insoluble
K <sup>+</sup>	39	1.33 (as nonhydrated)	1.917 as KCl [7]	do
Ethanol	46	2.4 <sup>a</sup>	1.24 [3]	0.02 [6]
Urea	60	2.64	1.38 [4]	0.00015 [5]
Antipyrine	188	4.1 <sup>b</sup>	0.68	0.012 [6]
Sucrose	342	5.55	0.57 [4]	Insoluble
Dextran-75 000	60 000-90 000	60	0.036 [5]	do

<sup>a</sup> Calculated as Stokes-Einstein radius  $r = RT/6\pi\eta DN$

<sup>b</sup> Calculated as equivalent sphere radius  $r = (3M/4\pi N\rho)^{1/3}$

(M is the molecular weight,  $\rho$  is the density,  $N$  is Avogadro's number,  $R$  is the gas constant,  $T$  is the absolute temperature and  $\eta$  is the viscosity of the solvent.)

Found by extrapolation from the diffusion coefficients of urea, glucose and sucrose

References: [1] Wang *et al.* 1953; [2] Handbook of Chemistry and Physics 1972; [3] Johnson and Babb 1956; [4] Longworth 1954; [5] Personal communication from New England Nuclear; [6] Collander and Bärilund 1933; [7] International Critical Tables 1929

Eel plasma was used in a few experiments. Blood was collected from the ventral aorta of 3-4 eels, heparinized, centrifuged and filtered in the same way as for horse plasma.

Papaverine sulphate was added to the perfusates (0.1 mg/ml blood) in order to achieve total papaverine is of the vasculature (Stray Pedersen 1970) and thereby obtain stable perfusion conditions.

In order to set up a gradient across the rete capillaries the substance under study was added to the arterial or to the venous perfusate. To maintain iso-osmotic conditions the addition of test molecules to one perfusate was balanced by addition of equimolar amounts of NaCl to the other perfusate.

#### Test substance

The capillary permeability of the following substances were studied

##### As isotopes

<sup>22</sup>Na (as NaCl, Institutt for Atomenergi, Kjeller, Norway), <sup>40</sup>K (as KCl, do.) and antipyrine <sup>14</sup>C (Catalogue number NEC 398, New England Nuclear, Boston, Mass.), urea <sup>14</sup>C (NEC 108, <sup>14</sup>N), sucrose-<sup>14</sup>C (NEC 100, <sup>14</sup>N), ethanol <sup>14</sup>C (NEC-079, <sup>14</sup>N), dextran-carboxyl <sup>14</sup>C (MW 60 000-90 000) (NEC-118, <sup>14</sup>N), and THO (Institutt for Atomenergi, Kjeller, Norway).

##### As non-isotopes

K (as KCl), Na (as NaCl), Cl (as NaCl)

Relevant physical data of the tracers are listed in Table I

#### Chemical analyses

The concentration of K<sup>+</sup> and Na<sup>+</sup> were measured with an Eppendorf flame photometer. The determination of K<sup>+</sup> and Na<sup>+</sup> was accurate to  $\pm 5\%$  at concentrations in the range of normal human plasma values. Cl<sup>-</sup> was determined with an accuracy of  $\pm 1\%$  on a chloride meter (electrical titration) (Fcl).

#### Radiocchemical analyses

A J Channel Auto Gamma Spectrometer and Tricarb Liquid Scintillation Spectrometer (Packard Instrument Co.) were used for the measurements of radioactivity. Precautions were taken in order to correct

the isotopes separately. To achieve high accuracy the activities in the reference solutions were adjusted when possible to give at least 10 000 c.p.m. in the output samples. As scintillation fluid was used Instagel (Packard Instrument Co.)

### Assumptions and Calculations

The capillary permeabilities were computed from the data of flow and concentrations of the test molecules in the input and output solutions obtained during steady state conditions of perfusion (steady state = the pressures, the input and output flow and the concentrations in the in- and output fluids being constant during the actual experimental period).

The output concentrations result from at least three different exchange processes

- 1 Bulk flow through arterio-venous shunts
- 2 Hydrodynamic flow caused by a transcapillary hydrostatic or osmotic pressure difference (filtration)
- 3 Diffusion

The analytical results must be corrected for the contribution from 1 and 2 before the data are used to calculate the diffusional permeability.

We recognized transcapillary bulk flow (shunts) as a difference between the concentration decline in one retial circuit compared to the concentration increase in the other. Quantitative estimate of shunts was based on the dextran 75 000 values. In experiments showing minimal leakages to the exterior bulk flow could also be detected by comparing the rate of output flow with that of the input. By optical inspection we observed only one location for the shunts, namely at the output (bladder pole) of the *rete*. In most cases it was caused by incomplete closing of the numerous anastomoses between the cannulated vessels in the bladder wall.

The quantitative extent of filtration was detected as a higher output than input concentration of dextran 75 000 in the circuit of the *rete* having the highest pressure.

#### Derivation of permeability equations

The mathematical formulas were derived from a single capillary model (Krogh 1919). The arterial and venous vascular systems of the *rete* were reduced to two coaxial cylinders of equal length, the surface of the inner cylinder representing the area available to capillary exchange (Fig. 2 and 3). The necessary symbols used in the derivation are listed in Fig. 3. Notice that during counter-current perfusion  $W_A = -W_V$ .

The formulas are valid only when the test substance is incompletely exchanged, i.e. when exchange is diffusion- and not flow-limited. As the arterial blood passes from  $x$  to  $x+dx$  (Fig. 3) an infinitesimal change in concentration of solute  $dc_{A,x}$  will take place due to exchange of solute over area  $A_C dx$ . Using Fick's 1st law of diffusion for this exchange and assuming that  $C_{A,0} > C_V$ , the mass balance equation for the arterial system will be

$$C_A x W_A - C_A x+dx W_A = -W_A dc_{A,x} - P A_C dx (C_A - C_V) \quad (1)$$

The corresponding equation for the venous system is

$$C_V W_V - C_V x+dx W_V = W_V dc_V - P A_C dx (C_A - C_V) \quad (2)$$

The difference  $C_A - C_V$  is called  $\Delta C_x$ , i.e.

$$\Delta C = C_A - C_V \quad (3)$$

Differentiating this equation with respect to  $dx$  gives

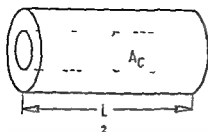
$$\frac{d(\Delta C)}{dx} = \frac{dc_A}{dx} - \frac{dc_V}{dx} \quad (4)$$

Combining Eqs. 1 and 4 and rearranging gives

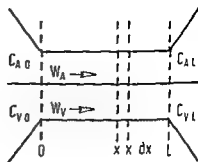
$$\frac{d(\Delta C)}{\Delta C} = -P A_C \left( \frac{1}{W_A} + \frac{1}{W_V} \right) dx \quad (5)$$

Integration gives

$$\ln(\Delta C) = -P A_C \left( \frac{1}{W_A} + \frac{1}{W_V} \right) x + \text{const.} \quad (6)$$



2



3

Fig. 2. Simplified model of the capillary systems in the *rete*. The arterial and venous capillary systems are reduced to two coaxial cylinders of equal length ( $L$ ). The circumferential area of the inner cylinder ( $A_C$ ) represents the circumferential area available to capillary exchange.

Fig. 3. Simplified model of the capillary systems in the *rete* (Same as in Fig. 2 the cylinders being sectioned longitudinally). List of symbols used in the figure and in the derivation of the permeability equations.

- $C_{A0}$  = arterial pre rete concentration (e.g. mol cm<sup>-3</sup>)  
 $C_{V0}$  = venous pre rete concentration (do)  
 $C_{AL}$  = arterial post rete concentration (do)  
 $C_{VL}$  = venous post rete concentration (do)  
 $W_A$  = arterial flow (cm<sup>3</sup> sec<sup>-1</sup>)  
 $W_V$  = venous flow (do)  
 $L$  = length of the *rete* (cm)  
 $A_C$  = circumferential area (cm) i.e. capillary surface area per unit path length  
 $P$  = permeability (cm sec<sup>-1</sup>)  
 $\Delta C_0$  = arterio-venous concentration difference pre rete  
 $\Delta C_L$  = arterio-venous concentration difference post rete  
 $\Delta C_A$  = difference between arterial pre rete and post rete concentrations  
 $\Delta C_V$  = difference between the venous pre rete and post rete concentrations.

When  $x=0$   $\Delta C_x = \Delta C_0$  and hence

$$\text{const} = \ln(\Delta C_0) \quad (7)$$

Which inserted in Eq. 6 gives

$$\ln(\Delta C_x) = \ln(\Delta C_0) - PA_C \left( \frac{1}{W_A} + \frac{1}{W_V} \right) x$$

or

$$\Delta C_x = \Delta C_0 \exp \left[ -PA_C \left( \frac{1}{W_A} + \frac{1}{W_V} \right) x \right] \quad (8)$$

$\Delta C_x$  therefore changes exponentially as  $x$  goes from 0 to  $L$ .

If the arterial and venous system is perfused in counter-current fashion at equal flow ( $W_A = W_V$ ) then  $\Delta C_x = \Delta C_0$  which means that the arterio-venous concentration difference will be the same at all points along the *rete* and the arterial and venous concentrations will change linearly with capillary length (Fig. 4).  $P$  can in the counter-current case at equal flow be calculated directly from Eqs. 1, 2 and 3. Setting  $dc_A = -dc_V$  and  $W_A = W_V$  we get

$$P = \frac{W}{A_C L} \frac{\Delta C_A}{\Delta C_L} \quad \text{or} \quad \frac{W}{A_C L} \frac{\Delta C_V}{\Delta C_L} \quad \text{Counter-current flow} \quad (9)$$

Knowing the concentration difference at the output side ( $\Delta C_L$ ) the permeability at parallel flow may be deduced from Eq. 8

$$\Delta C_L = \Delta C_0 \exp \left[ -PA_C L \left( \frac{1}{W_A} + \frac{1}{W_V} \right) \right] \quad (10)$$

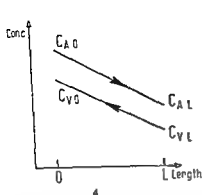


Fig. 4 Theoretical curves showing the change in arterial and venous concentrations in the *rete* as a function of the capillary length during counter-current perfusion at equal flow. The symbols have the same meanings as in Fig. 3.

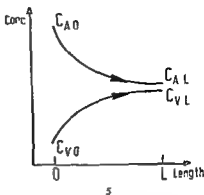


Fig. 5 Theoretical curves showing the change in arterial and venous concentrations in the *rete* as a function of the capillary length during parallel perfusion at equal flow. The symbols have the same meanings as in Fig. 3.

or

$$P = \frac{\ln \frac{\Delta C_0}{\Delta C_L}}{A_C L \left( \frac{1}{W_A} + \frac{1}{W_V} \right)} \quad (11)$$

If  $W_A = W_V$  as was the case in our experiments

$$P = \frac{W}{2A_C L} \ln \frac{\Delta C}{\Delta C_L} \quad \text{Parallel flow} \quad (12)$$

Fig. 5 shows the arterial and venous concentrations versus capillary length during parallel perfusion at equal flow.

The diffusion coefficient ( $D$ ) is equal to  $P \Delta x$ , where  $\Delta x$  is the thickness of the barrier separating the 2 capillary systems.  $P$  has the units of  $\text{cm sec}^{-1}$ ,  $D$  of  $\text{cm}^2 \text{sec}^{-1}$ .

The anatomical area ( $A$ ) and the thickness ( $\Delta x$ ) can be measured on fixed *etna*. The actual size of the diffusional area and barrier thickness in the experiments, however, was seen to be highly dependent on the perfusion conditions. Only in exceptional cases were the entire *rete* perfused. The diffusional area of partially perfused *rete* could be estimated by inspection of organs which had been fixated after and an ink injection at the end of the experiment. This was done in some cases. Microscopic inspection of cross sections indicated that perfused capillaries were predominantly found in bundles and not scattered throughout the *rete*. Normally about 60% of the capillaries were perfused. The true area was therefore less than the anatomical area, whereas the anatomical distance between arterial and venous capillaries could correctly be used as arterio-venous diffusional distance.

In the majority of the experiments the actual area available to transcapillary exchange was not measured. We therefore calculated the permeability surface products ( $PS$ ) = the capillary diffusion capacities (Renkin 1959 and 1968) for the test molecules

$$PS = W \frac{\Delta C_A}{\Delta C_L} = W \frac{\Delta C_V}{\Delta C_L} \quad (13)$$

in counter-current flow

$$PS = \frac{W}{2} \ln \frac{\Delta C}{\Delta C_L}$$

in parallel flow

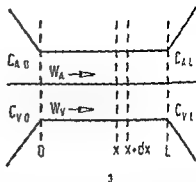
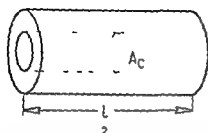


Fig. 2. Simplified model of the capillary systems in the rete. The arterial and venous capillary systems are reduced to two coaxial cylinders of equal length ( $L$ ). The circumferential area of the inner cylinder ( $A_C$ ) represents the circumferential area available to capillary exchange.

Fig. 3. Simplified model of the capillary systems in the rete (Same as in Fig. 2 the cylinders are sectioned longitudinally). List of symbols used in the figure and in the derivation of the permeability equations.

- $C_{A0}$  = arterial pre rete concentration (e.g. mol cm<sup>-3</sup>)  
 $C_{V0}$  = venous pre rete concentration (do)  
 $C_{AL}$  = arterial post rete concentration (do)  
 $C_{VL}$  = venous post rete concentration (do)  
 $W_A$  = arterial flow (cm<sup>3</sup> sec<sup>-1</sup>)  
 $W_V$  = venous flow (do)  
 $L$  = length of the rete (cm)  
 $A_C$  = circumferential area (cm) i.e. capillary surface area per unit path length  
 $P$  = permeability (cm sec<sup>-1</sup>)  
 $\Delta C_0$  = arterio-venous concentration difference pre rete  
 $\Delta C_L$  = arterio-venous concentration difference post rete  
 $\Delta C_A$  = difference between arterial pre rete and post rete concentrations  
 $\Delta C_V$  = difference between the venous pre rete and post rete concentrations

When  $x=0$   $\Delta C_x = \Delta C_0$  and hence

$$\text{const} = \ln(\Delta C_0) \quad (7)$$

Which inserted in Eq. 6 gives

$$\ln(\Delta C_x) = \ln(\Delta C_0) - PA_C \left( \frac{1}{W_A} + \frac{1}{W_V} \right) x$$

or

$$\Delta C_x = \Delta C_0 \exp \left[ -PA_C \left( \frac{1}{W_A} + \frac{1}{W_V} \right) x \right] \quad (8)$$

$\Delta C_x$  therefore changes exponentially as  $x$  goes from 0 to  $L$ .

If the arterial and venous system is perfused in counter-current fashion at equal flow ( $W_A = W_V$ ) then  $\Delta C_x = \Delta C_0$  which is to say that the arterio-venous concentration difference will be the same at all points along the rete and the arterial and venous concentrations will change linearly with capillary length (Fig. 4).  $P$  can in the counter-current case at equal flow be calculated directly from Eqs. 1 and 3. Setting  $dA = dC_V x$  and  $W_A = W_V$  we get

$$P \sim \frac{W}{A_C L} \frac{\Delta C_A}{\Delta C_L} \quad \text{or} \quad \frac{W}{A_C L} \frac{\Delta C_V}{\Delta C_L} \quad \text{Counter-current flow} \quad (9)$$

Knowing the concentration difference at the output side ( $\Delta C_L$ ) the permeability at parallel flow may be deduced from Eq. 8

$$\Delta C_L = \Delta C_0 \exp \left[ -PA_C L \left( \frac{1}{W_A} + \frac{1}{W_V} \right) \right] \quad (10)$$

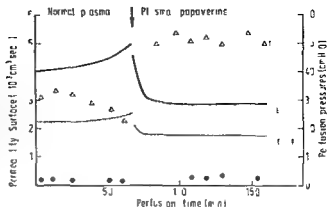


Fig. 7 The effect of papaverine on the capillary exchange. The curves and marks show the pressures and exchange obtained before and after addition of 10 mg/100 ml of papaverine to the perfusate. Thick line represents the arterial pressure, thin line the venous pressure.  $\Delta = P_{S\text{THO}}$ ,  $\bullet = P_{S\text{K}}$ .

The low values for capillary exchange obtained when the flow was small was most probably due to scattered perfusion of the *rete* with concomitant reduced area and increased path of diffusion. The increasing exchange at increasing flow is ascribed to a recruitment of new capillaries earlier unperfused. At flow rates above a certain level the permeability ratios were constant which indicated that the diffusional path was now reduced to the minimal one (mainly capillary membranes). On the basis of a series of such flow-experiments it appeared that the achievement of optimal exchange conditions was more related to the perfusion pressure than to the flow. Thus when the arterial perfusion pressure was 30 cm H<sub>2</sub>O or higher the  $P_{\text{THO}}/P_{\text{K}}$  ratio was maximal and the distribution of indian ink in the capillaries was even.

#### Effect of papaverine

The effect of papaverine on the capillary exchange was studied in 5 expts. As can be seen from Fig. 7 the pressure as well as the exchange was unstable during perfusion with normal plasma. The exchange was low and the ratio between the permeabilities of THO and K<sup>+</sup> about 20. When papaverine-containing perfusates had been introduced the pressures fell abruptly and then remained constant. The exchange of both substances however increased proportionally. Similar results were obtained in the other experiments. Papaverine therefore does not act on the permeability properties of the capillaries but contributes to more optimal perfusion conditions by paralyzing the smooth muscles of the retial vessels.

#### Effect of metabolic inhibitors

The *rete* was perfused with normal perfusate until steady state conditions had prevailed for 30 min. Then plasma containing 20 mM/l iodoacetate or/and 20 mM/l KCN was introduced. This further perfusion lasted for at least one hour in one experiment for 2 1/2 h. As test substances were used THO, K<sup>+</sup> and ethanol.

3 expts of this type were performed. The results from a typical experiment are shown in Fig. 8. In none of these experiments did the introduction of KCN/iodoacetate lead to significant changes in perfusion pressure or capillary exchange. The poisoned *retia* were examined histologically and no morphological changes could be observed.



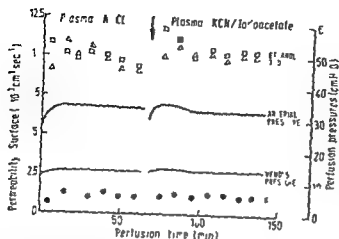


Fig. 8 The effect of metabolic poisons on the capillary exchange. The curves and marks represent the data for pressures and exchange obtained and after addition of  $\pm 0$  mM/l of KCN and 0 mM/l of iodoacetate to the perfusates during a perfusion. Thick line represents the arterial pressure, thin line the venous pressure.  $\square = PS_{\text{exchange}}$ ,  $\Delta = PS_{\text{THO}}$ ,  $\circ = PS_{\text{KCN}}$ .

### Capillary filtration

The capillary hydraulic conductivity was estimated from the increase in the concentration of the non permeating substance dextran 75 000 in the high pressure circuit of the *rete*.

The hydraulic conductivity ( $L_p$ ) was calculated according to the equation

$$L_p = \frac{v_f}{\Delta P \cdot A} \quad (15)$$

in which  $v_f$  represents the volume filtered ( $\text{cm}^3 \text{sec}^{-1}$ ),  $\Delta P$  the transcapillary hydrostatic pressure difference ( $\text{cm H}_2\text{O}$ ) and  $A$  the capillary area ( $\text{cm}^2$ ).  $L_p$  has the units of  $\text{cm}^3 \text{cm}^{-2} \text{sec}^{-1} \text{cm H}_2\text{O}^{-1}$ .

The capillary area ( $A$ ) was not measured in these experiments but estimated on the basis of the exchange of THO (See Methods).  $\Delta P$  was created by raising or lowering the output tubes and estimated from the equation

$$\Delta P = \frac{P_{A0} - P_{AL}}{2} - \frac{P_{V0} - P_{VL}}{2} \quad (16)$$

in which the subscripts 0 and L refer to the input and output side of the *rete*.

Since it was not possible to establish a higher pressure in the venous system, only capillary filtration in the arterio-venous direction could be measured.

Four successful experiments of this type were performed. The arterial output pressure was increased by 10–20 cm H<sub>2</sub>O which led to an increase in the transcapillary hydrostatic pressure of 7–16 cm H<sub>2</sub>O followed by a net arterio-venous volume flow. The size of this flow was about 2% of the input flow.

Series of samples were taken during the filtration. Using the area value estimated from the exchange obtained for THO, the arterio-venous hydraulic conductivities fell within the range of  $3.1\text{--}7.1 \times 10^{-7} \text{ cm}^3 \text{cm}^{-2} \text{sec}^{-1} \text{cm H}_2\text{O}^{-1}$  (Table II). The average value was  $4.2 \times 10^{-7} \text{ cm}^3 \text{cm}^{-2} \text{sec}^{-1} \text{cm H}_2\text{O}^{-1}$ . Since 1 cm H<sub>2</sub>O difference of (osmotic) pressure is equal to  $7.23 \times 10^{-7} \text{ g/cm}^3$  difference of water concentration (the density of water  $\approx 1.0 \text{ g/cm}^3$ ), this value corresponds to an hydraulic permeability ( $P_f$ ) of  $0.58 \text{ cm sec}^{-1}$ .

TABLE II Net volume flow due to capillary filtration and the corresponding hydraulic conductivities obtained in a series of filtrations (4 expts)

Exp	Series of filtration	Input flow $\mu\text{l}/\text{min}$	Transcapillary hydrostatic pressure difference $\Delta P$ cm $\text{H}_2\text{O}$	Capillary filtration <sup>a</sup> $\bar{v}_f$ $\mu\text{l}/\text{min}$	Capillary area <sup>b</sup> $A$ $\text{cm}^2$	Capillary hydraulic conductivity $L_p$ $\text{cm}^3 \text{cm}^{-2} \text{sec}^{-1} \text{cm H}_2\text{O}^{-1} \times 10^7$
I	1	100	7	3.0	2	3.3
	3		16	14.4		6.4
	4		16	8.6		4.1
II	1	100	8	4.2	6	4.0
	1		9	4.6		3.3
	1		9	4.9		3.5
III	1	375	11	7.5	27	4.2
	2		12	6.8		3.5
IV	1	100	8	3.4	1	3.4
	2		13	5.9		3.6
	3		14	12.5		7.1

<sup>a</sup> Mean value of at least 3 measurements<sup>b</sup> Estimated from the capillary exchange of THO

In no experiment did the concentration of  $\text{K}^+$  urea or sucrose increase in the arterial system during a capillary filtration

#### The osmotic effect of albumin

An arterio-venous hydrostatic pressure difference of about 10 cm  $\text{H}_2\text{O}$  lead to a measurable rate of capillary filtration in the *rete*. We would therefore expect a measurable osmotic flow of water when similar differences in osmotic pressure were imposed upon the system

The osmotic effect of albumin (bovine albumin VI Sigma) was studied in 2 expts. The behaviour of dextran 75 000 during filtration suggests that the retial capillaries are virtually impermeable to albumin (MW 60 000). According to the empirical equation describing the osmotic pressure-concentration curves for albumin solutions (Landis and Pappenheimer 1963) an addition of 6 g/100 ml (1 mM/l) of albumin to one of the perfusates would create an osmotic pressure difference of about 35 cm  $\text{H}_2\text{O}$ . The albumin-containing perfusate served alternatively as arterial or venous input. As test substances were used THO, K<sup>+</sup> sucrose and dextran 75 000. These substances were added either to the albumine perfusate or to the other

The ratio between the viscosity of albumin plasma and that of normal plasma was found to be 1.43 by measurements of the perfusion pressures of these solutions through narrow polyethylene catheters. The introduction of albumin was consequently followed by an increase of the perfusion pressure. This pressure increase could be observed in both systems especially when albumin was added to the venous perfusate

Similar to the measurements of capillary filtration the osmotic flow was estimated from the data of dextran 75 000 and/or the output flow. In order to calculate the osmotic conductivity ( $L_{pD}$ ) the following equation was used

$$P_{\text{osm}} = \frac{v_{\text{osm}}}{(\Delta\pi - \Delta P) A} \quad (17)$$

in which  $v_{osm}$  represents the osmotic flow ( $\text{cm}^3 \text{sec}^{-1}$ ),  $\Delta\pi$  the transcapillary osmotic pressure difference ( $\text{cm H}_2\text{O}$ ) estimated according to van t Hoff's law assuming  $\sigma = 1$  and  $\Delta P$  the transcapillary hydrostatic pressure difference calculated from Eq. 16 ( $\text{cm H}_2\text{O}$ ). The capillary area ( $A$ ) was estimated from the exchange of THO obtained in the experiments.  $L_{pD}$  has the same units as the capillary hydraulic conductivity ( $\text{cm}^3 \text{cm}^{-2} \text{sec}^{-1} \text{cm H}_2\text{O}^{-1}$ ).

Two experiments including 6 measurements of the osmotic flow were performed. The osmotic conductivities were between  $1.7\text{--}5.8 \times 10^{-7} \text{ cm}^3 \text{cm}^{-2} \text{sec}^{-1} \text{cm H}_2\text{O}^{-1}$  with an average of  $2.4 \times 10^{-7} \text{ cm}^3 \text{cm}^{-2} \text{sec}^{-1} \text{cm H}_2\text{O}^{-1}$ . This corresponds to a  $P_{osm}$  of  $0.33 \text{ cm sec}^{-1}$ . The osmotic permeability in the arterio-venous direction did not differ significantly from that obtained in the opposite direction.  $\text{K}^+$  and sucrose were not concentrated as a consequence of the osmotic flow.

#### *The osmotic effect of NaCl, urea and sucrose*

Since the *rete* capillaries are 50–100 times less permeable to NaCl, urea and sucrose than to water, one would expect that these substances would show osmotic activity. In three experiments 25 mM/l of NaCl, urea or sucrose was added to the arterial or the venous perfusate. Assuming  $\sigma = 1$  for these molecules this would give a transcapillary osmotic pressure difference of several hundred  $\text{cm H}_2\text{O}$ .

2 expts. of this type were performed. THO,  $\text{K}^+$ , urea, sucrose and dextran 75 000 served as test molecules. In these experiments the concentrations of Na<sup>+</sup> and Cl<sup>-</sup> were also measured. One set of experimental data obtained is shown in Table III.

As may be seen from the table the addition of sucrose, NaCl or urea was not followed by any significant changes in flow, perfusion pressure or capillary exchange. The increase in the arterial perfusion pressure was seen to take place continuously and was not related to the onset of the different perfusion. The gain in venous outflow throughout the experiment was caused by an arterio-venous shunt on the output side, as indicated from the data of sucrose and  $\text{K}^+$ .

In the second experiment these substances were added to the zero-perfusate (containing no test-substances) which was used as venous input. During none of the perfusions dextran 75 000, sucrose, urea, Na<sup>+</sup>, Cl<sup>-</sup> or  $\text{K}^+$  was seen to accumulate in the arterial output, thus clearly indicating that these substances were osmotically inactive to the capillary membrane.

#### *The tracer permeability of the rete capillaries*

The capillary exchange varied considerably from one preparation to another. These variations were obviously related to differences in the actual capillary area between the preparations. This again could partly be due to differences in the perfusion conditions, but also to the variations of the sizes of the *retia* employed.

The area available to capillary exchange was estimated by the indian ink method on four *retia* which, as judged from our criteria, yielded optimal experimental conditions. Table IV lists the capillary area, the THO and the  $\text{K}^+$  permeability constants obtained in these organs. The average value of  $P_{THO} = 33.2 \times 10^3 \text{ cm sec}^{-1}$  therefore represents the best approximation to the true permeability of the retial capillaries.

In about 90 other perfusion experiments the capillary area was not measured. The capillary

TABLE III The osmotic effects of NaCl, urea and sucrose (Data from 1 expt.) Input flow 0.2 ml/min. The table lists the data obtained in an experiment consisting of a series of plasma perfusions in which isotonic NaCl was continuously added to the venous perfusate whereas either isotonic NaCl, 25 mM/l sucrose, 25 mM/l NaCl or 25 mM/l urea was added to the arterial perfusate. Abbreviations: Inp A = arterial input, Inp V = venous input, Outp A = arterial output, Outp V = venous output.

Perfusions	Input pressures (cm H <sub>2</sub> O)	Flow (in of input flow)	Na <sup>+</sup> mE/l	Cl <sup>-</sup> mE/l	THO c.p.m. ( $\times 10^{-3}$ )	K <sup>+</sup> mE/l	Sucrose activity c.p.m. ( $\times 10^{-3}$ )
I							
Inp A Isotonic NaCl	41	100	127	10	260	6.87	4.9
Inp V Isotonic NaCl	21	100	131	106	0	2.91	0.2
Outp A		91.2	127	101	146	6.78	4.7
Outp V		104.8	118	103	118	3.18	2.4
II							
Inp A 25 mM/l sucrose	47	100	11	89	261	6.59	4.5
Inp V isotonic NaCl	19	100	131	106	0	2.91	0.2
Outp A		91.6	113	91	151	6.48	4.0
Outp V		104.6	127	104	117	3.18	2.4
III							
Inp A 25 mM/l NaCl	44	100	148	130	261	6.59	4.6
Inp V isotonic NaCl	19	100	131	106	0	2.91	0.2
Outp A		91.0	141	123	154	6.50	4.2
Outp V		104.7	129	108	118	3.14	2.4
IV							
Inp A 25 mM/l urea	47	100	110	90	258	6.60	4.0
Inp V isotonic NaCl	19	100	131	106	0	2.91	0.2
Outp A		91.5	113	9	152	6.48	4.4
Outp V		104.7	119	103	117	3.15	2.3
V							
Inp A isotonic NaCl	48	100	123	102	60	6.88	4.2
Inp V isotonic NaCl	19	100	131	106	0	2.91	0.2
Outp A		90.2	124	103	160	6.80	4.2
Outp V		104.6	131	105	12	3.18	2.5

diffusion capacities (or PS products) of the different test molecules were therefore related to that of the reference molecules THO and K<sup>+</sup> and present in Table V as permeability ratios.

In 14 of these expts. there was no evidence of capillary filtration and the volume flow due to shunts or leakages was less than 5% of the input flow (ideal experiments). As demonstrated in Table V the permeability ratios  $P_{\text{THO}}/P_{\text{K}^+}$  were uniform and similar to those from the four experiments mentioned above.

The remaining 74 expts. suffered from various shortcomings and showed lower exchange ratios. However, as seen from Table V, the general permeability pattern was not essentially different. These experiments also include all of counter-current perfusions and the 3 expts. with eel plasma as perfusate.

The transport of K<sup>+</sup> was found to be similar to that of (cold) K<sup>+</sup>. It was of no significance to the capillary exchange whether the test substances were added to the arterial or to the venous perfusate.

TABLE VII Hydraulic (osmotic) permeabilities ( $P_s$ ) tracer permeabilities of water ( $P_w$ ) and equivalent pore radius ( $r_p$ ) of different biological membranes

Membrane	$P_s$ ( $\times 10^8$ cm sec $^{-1}$ )	$P_w$ ( $\times 10^8$ cm sec $^{-1}$ )	$P_s/P_w$	$r_p^a$ (Å)	References
Man: rete capillaries	11 000	36 <sup>c</sup>	1610 <sup>d</sup>	167 <sup>f</sup>	Present study
Cat: skeletal muscle capillaries	4 000 <sup>b</sup>	28 <sup>d</sup>	150	48	[1]
Rat: kidney proximal tubules	1 000	560	38	74	[1]
Toad: bladder (with vasopressin)	1 880	16	118	40	[3]
Frog: ovarian egg	891	12.8	70	30	[4]
Human erythrocyte	1 770	550	74	3.5	[5]

<sup>a</sup> Computed according to the relation quoted below (Eq. 18)

<sup>b</sup> Calculated on the assumption of a capillary surface area of 7 000 cm $^2$ /100 g muscle

<sup>c</sup> Extrapolated from the tracer permeability of THO

<sup>d</sup> Extrapolated from ( $A_p/x$ ) of NaCl

( $\eta$  = viscosity of water  $D$  = free diffusion coefficient of water

$V$  = molar volume of water  $R$  = gas constant  $T$  = absolute temperature)

Estimated on data from simultaneous measurements

<sup>f</sup> This value does not represent the intercellular pore radius (see text)

References: [1] Lands and Pappenheimer 1953 [2] Persson and Ulfendahl 1970 [3] Paganelli and Solomon 1957 [4] Prescott and Zeuthen 1953 [5] Sjolin 1954

These calculations were performed as follows:

If we assume the capillary pores to be much wider than the radius of the water molecule, the pore radius ( $r_p$ ) may be obtained from the following relation (Paganelli and Solomon 1957):

$$\text{Eq. 18} \quad r_p^2 = (P_s/P_w - 1) (8n_w D_w V_w / RT)$$

in which  $n_w$  represents the viscosity,  $D_w$  the free diffusion coefficient,  $V_w$  the molar volume of water,  $R$  the gas constant, and  $T$  the absolute temperature. At 37°C the factor  $8n_w D_w V_w / RT = 14.5 \cdot 10^{-10}$  cm $^2$ .

The tracer permeability of water through the pores was obtained by extrapolation from  $P_K$  measured during the filtration experiments where  $L_p$  was estimated (Table II).

The area and the frequency of pores were calculated under the assumption of a functional capillary barrier of the type constituting 50% of the anatomical surface and having a thickness of 1.6  $\mu$ m (Stray Pedersen and Nicolayson 1973).

We are aware of the questionable applicability of Poiseuille's law to flow through tortuous fibrous channels, but also aware of the fact that laminar flow is less energy requiring than turbulent. This method gives therefore the minimum pore size that can explain a flow of the magnitude measured in the pressure differences employed.

We are therefore confronted with the following contradiction: The hydrodynamic flow measured through the degree of accumulation of a 100 Å molecule (dextran 75 000) yields a pore diameter 13 times larger. We suggest that the *in vivo* intercellular junction is about 1500 Å in diameter, but filled with fibrous intercellular material. This material will contribute little resistance to the hydrodynamic flow, but constitute an effective molecular sieve.

An immediate objection to this model is that intercellular junctions of this size have

never been observed. We notice however that cell junctions have never been observed *in vivo* but only after fixation. This procedure may well change the ultrastructure of junctions as much as freezing changes the structure of water. Furthermore capillary beds which are made readily permeable to dextran 75 000 by removal of  $\text{Ca}^{++}$  with EDTA show no changes in the junctional structure (Stray Pedersen 1975). This has also been reported for the rabbit lung showing fulminant edema due to treatment with EDTA (Hovig *et al* 1971). The junctions of the *rete* still appear as channels no wider than 100–120 Å though significantly permeable to a 100 Å molecule.

Admittedly our perfusion experiments did not allow precise estimations of the hydraulic conductivity (or  $P_f$ ). The values obtained varied from experiment to experiment. We therefore suggest the  $P_f$ -value quoted in Table VII to be accurate by a factor of 2 but comparable to the  $P_f$  of intestinal mucosa capillaries of rats (Folkow *et al* 1963).

Our high  $P_f$ -values could be due to widening of the pores as a consequence of distention of the capillary wall when the pressure was increased. The presence of such stretched pore phenomenon in capillaries has been discussed but it seems doubtful if this phenomenon is present *in vivo* (Zweifach and Intaglietta 1968). In our experiments the capillary exchange of the test molecules seemed to remain unchanged during a filtration or osmotic flow. The possibility might exist however that some pores could be substantially widened. This would predominantly affect the size of the hydrodynamic flow since this depends on the fourth power of the pore radius whereas diffusional flow is related to the second power of the radius.

The *rete* permeability constants and consequently also the diffusion constants of lipid insoluble molecules—except for THO—seem to be considerably smaller than those reported from other capillary beds (Table VI). However we have to consider that other workers may have great difficulties in estimating the actual capillary area as well as the diffusion distance in the organ investigated. Pappenheimer *et al* (1951) assumed a capillary area of 7000 cm<sup>2</sup> per 100 g of muscle and a wall thickness of 1  $\mu$ m in order to calculate the permeability and diffusion constants in skeletal muscle capillaries. In the *rete* the diffusional path length may vary tremendously from 0.5  $\mu$ m up to several  $\mu$ m with an average of about 2  $\mu$ m. As judged from cross-sections of the *rete* it would be reasonable to suggest that for the greatest fraction of transcapillary transport would take place across an arterio-venous barrier with an average thickness of 1.6  $\mu$ m constituting about 50% of the circumference of the capillaries (Stray Pedersen and Nicolaysen 1975). If we consider this barrier to represent the functional arterio-venous membrane, then the retal capillary permeability (and diffusion constant) to for instance sucrose would be very similar to that obtained by Crone (1963) in the skeletal muscles of dog and by Alvarez and Yudilevich (1969) in the dog heart (Table VI).

From the direct observations of transcapillary transport as for instance reported by Karnovsky (1967), Schneeberger-Keeley and Karnovsky (1968) and Michel (1970) there seems to be no doubt that the intercellular junctions serve as transport channels to hydrophilic molecules. However the discrepancy between the pore dimensions calculated on the basis of permeability studies and those actually observed on electron micrographs (present study, Faber and Stearns 1969, Boyd *et al* 1969, Faber *et al* 1971, Normand *et al* 1971,

makes it tempting to suggest the existence of alternative transport pathways to these molecules. In the dog brain it has been demonstrated that transcapillary transport of sugar molecules most probably takes place through the endothelial cells by facilitated diffusion mechanisms (Crone and Thompson 1973). Bruns and Palade (1968) have demonstrated that macromolecules such as ferritin (diam  $\sim 110$  Å) are transported via the pinocytotic vesicles. From kinetic studies it seems to be established that the vesicular system at least could represent "the large pore system" of capillaries (Shea *et al* 1970). The presence of giant vacuoles and cytoplasmic extrusions (microvilli) in the *rete* endothelial cells (Stray Pedersen and Nicolaysen 1975) may indicate that transcapillary vacuolar transport may also occur in these capillaries. Vesicular or vacuolar transport are generally assumed to take place by Brownian movements which are obviously too slow to account for the rapid exchange of for instance the ions. As suggested by Bruns and Palade (1968) however the possibility might exist that vesicles may become fused and thereby form continuous chains through the endothelial cells. In the *rete* small chains of 2-3 intercommunicating vesicles represented a rather common finding (Stray Pedersen and Nicolaysen 1975). Even though continuous chains in the luminal-abluminal axis were never observed it can not be excluded that such chains may exist *in vivo*.

It might also be that the vesicles (and vacuoles?) which appear as isolated structures on electron micrographs in fact represent sections of tortuous channels or tubules which are continuous throughout the cell. This hypothesis has recently been proposed by Bendayan *et al* (1974) on the basis of ultrastructural studies of the *retial* endothelial cells. According to these investigators the alternate dilatations and constrictions of the tubules observed on the micrographs may indicate the tubules to exhibit peristaltic activity. A transcapillary transport through a system of contractile tubules may be considerably more rapid and require less metabolic energy than transport via pinocytotic vesicles. The diameter of the dilated parts of the tubules was found to be as great as 1300 Å which conforms precisely to the value for the dimension of the *retial* capillary pore calculated in the present investigation.

Finally we would like to offer an alternative explanation to the experimental results of Pappenheimer *et al* (1951) which founded the single pore theory. We believe that when plasma is made hyperosmotic with e.g. NaCl an osmotic dehydration of the cells is induced. The simultaneously induced increase in venous pressure (in order to achieve isogravimetric conditions) will cause flow of plasma filtrate out through the junctions and into the extra vascular space. With time NaCl-diffusion into the cells will establish a new equilibrium. When the plasma is made hyperosmotic by e.g. inulin the osmotic effect would be both across the endothelial cell membranes and across the junctions. We shall be aware however that the osmotic behaviour of membranes with more than one population of pores available to the diffusion of water is poorly understood.

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## The Effect of $\text{Ca}^{++}$ , $\text{Mg}^{++}$ and $\text{H}^+$ on the Capillary Permeability of the *rete mirabile* of the Eel, *Anguilla vulgaris* L

By

SVERRE STRAY PEDERSEN

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### Abstract

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STRAY PEDERSEN S: The effect of  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  and  $\text{H}^+$  on the capillary permeability in the *rete mirabile* of the eel *Anguilla vulgaris* L. Acta physiol scand 1975 94 423-441

The effects of EDTA and of varying pH on the capillary permeability in the *rete mirabile* have been investigated. EDTA had to be added to the perfusate in excess to the total content of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  in plasma in order to elicit changes in the permeability. When exposed to sufficient amounts of EDTA the capillary permeability to hydrophobic molecules increased several fold whereas the permeability to THO and lipid-soluble molecules remained unchanged. The increase in the permeability to high molecular dextrans was delayed. When the EDTA-effect was maximal the mutual relationship between the permeabilities of THO and the lipid insoluble molecules became close to the ratio between the corresponding free diffusion coefficients in water. During exposure to EDTA the content of  $\text{K}^+$  and  $\text{Na}^+$  of the *rete* tissue (mostly endothelial cells) became very similar to that of plasma. Similar effects were obtained when the *rete* was perfused with plasma having a pH below 4 or above 9. The EDTA-effect was reversible during its initial stage. Perfusion with plasma in which  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  had been removed by dialysis at pH 7.5 was performed. When an EDTA-effect had been induced with this type of perfusates it could be reversed by addition of equimolar amounts of either  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$ . This indicates  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  to be equally important in order to assure normal permeability properties of the capillary membrane.

The importance of calcium for the maintenance of normal capillary permeability was first demonstrated by Chambers and Zweifach (1940) on frog mesenterium. Wilbrandt *et al* (1956) found that addition of EDTA induced a marked reduction in the effective osmotic pressure. An increase of local capillary permeability was observed by Spector (1957) when sodium citrate was injected into the skin of rats. Nikolaysen (1971 a, b) found that the prevention of edema development in rabbit lungs was determined by either the  $[\text{Mg}^{++}]$  or the  $[\text{Ca}^{++}] + [\text{Mg}^{++}]$ . Increased hydraulic conductivity was observed at low  $[\text{Ca}^{++}]$ , this effect could be reversed by  $\text{Ca}^{++}$  but not by  $\text{Mg}^{++}$ .

A reduction of  $[\text{Ca}^{++}]$  (and  $[\text{Mg}^{++}]$ ) causes a marked increase in the ion permeability in various types of tissue membranes (Curran 1961, Lipson *et al* 1965, Tidball 1964, Goldner 1967, Cuthbert and Maetz 1972) and of cells (Lyman 1945, Maizels 1956, Bolingbroke and Maizels 1959, Morill *et al* 1965, Morill and Robbins 1967). Addition of  $\text{Ca}^{++}$  usually

leads to recovery but there seems to be some disagreement about the role of  $Mg^{++}$  as a replacement ion for  $Ca^{++}$ .

The site of action of low  $[Ca^{++}]$  on cellular membranes is not precisely known. Most investigators call attention to the junctions between the epithelial or endothelial cells. With drawal of  $Ca^{++}$  it is said to weaken the cell adhesions (Chambers and Zweifach 1947 Hays *et al.* 1965 Loewenstein 1971). Goldner (1967) however believes that the increased permeability caused by the exposure to EDTA is due to changes of the membranes of the epithelial cells rather than to changes at the cell junctions.

No informations about the effect of changes in pH on the capillary permeability seem to exist. The permeability of cells however has been shown to be influenced by variations of the hydrogen ion concentration in the extracellular fluid. A loss of  $K^+$  from the sartorius muscle of frog to the incubation fluid caused by an increase in  $pCO_2$  has been described by Fenn and Cobb (1934 and 1935). In the gall bladder epithelium a decrease or an increase in pH induce changes in the selective permeability to monovalent ions (Wright and Diamond 1968).

In the present investigation the effects of added EDTA and of pH variations on the capillary permeability have been studied. To this end horse plasma perfusions of the swimbladder *rete mirabile* of the eel have been performed. In this preparation a steady state transcapillary exchange could be established and the capillary permeability of various test substances continuously followed (Stray Pedersen and Steen 1973). The reversibility of the effects induced by EDTA or changes in pH was also investigated likewise the relative importance of  $Ca^{++}$  and  $Mg^{++}$ . The results obtained in this investigation throw some light on the mechanisms of capillary permeability particularly of the function of  $Ca^{++}$  and  $Mg^{++}$ . In this respect

## Methods

### *The preparation and perfusion technique*

For details the reader is referred to the papers of Stray Pedersen and Nicolaysen (1973) and of Stray Pedersen and Steen (1973).

Cannulation of the appropriate vessels allowed simultaneous perfusion of the arterial and venous capillaries of the isolated *rete*. The preparation was perfused by horse plasma to which the substances under study were added. Permeability values were obtained by comparing the concentrations of these substances in the output with those in the input perfusates.

The *rete* was always perfused in a parallel fashion at constant flow (Unita Perfusionum - Braun Melsungen). The accuracy of the delivery of the pumps was better than 0.2%. The perfusion pressures in both input cannulas were continuously recorded by connecting a side branch of the cannulas to a Statham P23 De transducer. Optimal perfusion of the organ is obtained at an arterial input pressure of about 40 cm  $H_2O$ . The flow was therefore adjusted to achieve this pressure. At such conditions the venous input pressure would be about 13 cm  $H_2O$ . The output flow was determined by weighing the output accumulated over known periods of time. Output samples of 1 ml were sufficient for analysis.

*Perfusate*. Horse plasma was obtained by centrifugation of heparinized blood (30 IU of Heparin (A.L.) per ml). Papaverine (0.1 mg/ml) was added to the perfusate in order to obtain a complete relaxation of the vascular smooth muscles (Stray Pedersen 1970). Papaverine in this dose does not affect the permeability of the *rete* capillaries (Stray Pedersen and Steen 1973).

To study the effect of  $Ca^{++}$  and  $Mg^{++}$  EDTA (as Na EDTA) was added to the perfusate in varying concentrations. At pH 7.4 the conditional constant of the  $Ca$  EDTA chelate is  $10^{-10}$  and of the  $Mg$  EDTA chelate  $10^{-11}$  (Ringbom 1967). Hence an addition of equimolar amounts of EDTA to plasma will lead to an almost complete removal of  $Ca^{++}$  and  $Mg^{++}$ .

TABLE I Relevant physico-chemical data of the test-substances

Substance	MW	Molecular (ionic) radius (Å)	$D_{25}$ ( $\times 10^5$ cm sec $^{-2}$ )	Olive oil/water partition coefficients
(Water)	18	1.5	2.66 [1]	0.007 [6]
THO	20	1.5	2.44 [1]	do (?)
Na $^{+}$	23	0.95 (as nonhydrated)	1.48 as NaCl [ ]	Insoluble in oil
K $^{+}$	39	1.33 (as nonhydrated)	1.917 as KCl [ ]	do
Ethanol	46	2.4	1.44 [3]	0.0006 [6]
Urea	60	2.64	1.38 [4]	0.00015 [6]
Antipyrine	188	4.1 <sup>b</sup>	0.68	0.03 [6]
Sucrose	342	5.55	0.5 [4]	Insoluble in oil
Dextran-16 000	16 000-17 000	28	0.074 [5]	do
Dextran 75 000	60 000-90 000	60	0.036 [5]	do

<sup>a</sup> Calculated as Stokes-Einstein radius  $r = RT/6\pi\eta D_N$

<sup>b</sup> Calculated as equivalent sphere radius  $r = (3M/4\pi N)^{1/3}$

(M is the molecular weight  $\rho$  is the density N is Avogadro's number R is the gas constant, T is the absolute temperature and  $\eta$  is the viscosity of the solvent)

<sup>c</sup> Found by extrapolation from the diffusion coefficients of urea, glucose and sucrose

References [1] Wang *et al.* 1953 [ ] Handbook of Chemistry and Physics 1971 [3] Johnson and Babb 1956 [4] Longworth 1954 [5] Personal communication from New England Nuclear [6] Collander and Bärklund 1933

Perfusates with low Ca/Mg-content were prepared by dialysis for 40-60 h against deionized water at +4°C following acidification to pH 3.0-3.5 by addition of concentrated HCl. To restore normal concentrations of Na $^{+}$ , K $^{+}$  and Cl $^{-}$  appropriate amounts of NaCl and KCl were added or the plasma was dialyzed against a Ca/Mg free Ringer solution. By this procedure plasma with less than 0.005 mM/l of Ca and less than 0.003 mM/l of Mg was obtained.

The effects of pH on the capillary permeability were studied by adding concentrated HCl and NaOH to the perfusates. At extreme pH values plasma would flocculate and repeated filtration was necessary prior to the experiment.

Test material The capillary permeability of the following substances were studied

#### Isotopes

$^{22}Na$  (as NaCl Institut for Atomenergi Kjeller Norway)

$^{42}K$  (as KCl, do)

Antipyrine  $N$ -methyl  $^{14}C$  (Catalogue number NEC 395 New England Nuclear Boston, Mass.)

Urea- $^{14}C$  (NEC 108 NEN)

Dextran carboxyl  $^{14}C$  MW 15 000-17 000 (NEC 18A, NEN)

Dextran carboxyl  $^{14}C$  MW 60 000-90 000 (NEC 18B, NEN)

Ethanol  $^{14}C$  (NEC-09 NEN)

THO (IFA Kjeller Norway)

Sucrose- $^{14}C$  (NEC 100 NEN)

#### Non Isotopes

K as KCl

Some relevant physical properties of the test molecules are listed in Table I

The substances to be investigated were added to one of the perfusates. Care was taken to maintain the same osmolarity in the arterial and venous perfusate. Hence addition of substances to one side was balanced by addition of NaCl or H $_2$ O to the other. K $^{+}$  and THO were used in all experiments to serve as reference molecules. Due to its easy determination, the capillary exchange of K $^{+}$  could be followed throughout the experiments.

**Radiochemical analysis** A 3 channel Auto Gamma Spectrometer and Tr Carb Liquid Scintillation Spectrometer (Packard Instrument Co) were used for the measurements of radioactivity. Precautions were taken to count the isotopes separately. To achieve high accuracy the activity in the test perfusates was adjusted when possible to give at least 10 000 c.p.m. in the output samples.

#### Measurements of Na, K, Ca and Mg

The concentrations of Na, K, and Ca were measured with an Eppendorf flame photometer. Within the range of normal plasma concentrations the determinations were accurate to within  $\pm 1\%$  for Na and K, and  $\pm 3\%$  for Ca. The measurements of Mg and in some instances also Ca were performed with an Unicam atomic absorption photometer. In routine performances of this method the concentrations could only be measured when  $[Ca] > 0.05$  and  $[Mg] > 0.05$  mM/l. When necessary samples were concentrated by evaporation whereby concentrations down to 0.005 and 0.005 mM/l of Ca and Mg respectively could be determined. By these methods the total concentration of the substances (ionized plus non-ionized) were measured.

#### Determinations of uric content of the rete tissue

Pieces of rete were removed and carefully squeezed on filter paper to absorb as much intravascular fluid as possible. After weighing a known quantity of sulphuric acid was added and the solutions were lightly heated until total disintegration of the tissue. The chemical measurements were performed in this solution after addition of 0.05 ml  $H_2O$ . The other rete of the swimbladder (not perfused during the experiment) served as controls. (For details see Stray Pedersen and Nicolaysen 1974).

#### Calculations of capillary permeability values

The basic assumptions and the evaluation of the permeability equations as well as the methods for the necessary corrections for shunts and leakages have been described earlier (Stray Pedersen and Steen 1975). Since these experiments were performed with parallel perfusion at conditions of equal flow the permeability equation used was

$$P = \frac{W}{A_C L} \ln \frac{\Delta C_0}{\Delta C_L} \quad (1)$$

in which  $P$  = capillary permeability (cm sec<sup>-1</sup>),  $W$  = flow rate (cm<sup>3</sup> sec<sup>-1</sup>),  $A_C$  = capillary circumference (cm),  $L$  = capillary length (cm),  $\Delta C$  = arterio-venous concentration difference of the test molecule at the input side,  $\Delta C_L$  = arterio-venous concentration difference at the output side.

Since the size of the capillary surface ( $S = A_C L$ ) was not measured in these experiments we used the  $PS$  product as a measurement of the capillary exchange

$$PS = P A_C L = \frac{W}{\Delta C} \ln \frac{\Delta C_0}{\Delta C_L} \quad (2)$$

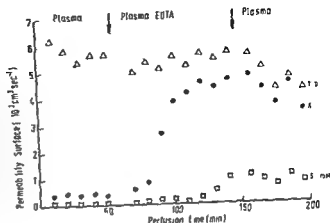
The  $PS$  products of the different test molecules were calculated according to this equation and related to those of THO or K as permeability ratios ( $P/P_{THO}$  or  $P/P_K$ ).

## Results

### The effect of Ca and Mg

The effect of different concentrations of EDTA in the perfusates was first studied. Perfusion with EDTA in concentrations of 10, 100, 150, 200 and 300% of the concentration of Ca and Mg in the plasma perfusates were performed. No alterations in the capillary exchange were observed until the concentration of EDTA exceeded the equimolar concentration of Ca + Mg. Even with 100% EDTA the permeability of all the test substances remained constant during more than 24 hours of perfusion. However with 120% EDTA or more a distinct effect on the permeability properties of the capillaries could be demon-

Fig. 1 The effect of EDTA. During the exposure to 6 mM EDTA both  $PS_{K^+}$  and  $PS_{Cl^-}$  increased whereas  $PS_{THO}$  remained fairly constant. Note the delay in the onset of the increase in  $PS_{Cl^-}$  compared to that of  $PS_{K^+}$ . At the end of the perfusion the mutual relationship between the capillary diffusion capacities was approximately identical in that between the corresponding free diffusion coefficients



strated. The latency for the effect to occur varied from experiment to experiment and seemed to be related to the rate of flow and the size of the *rete*. With 120% EDTA the effect would usually appear within 30 to 90 minutes. When higher concentrations of EDTA were used the permeability changes would develop more quickly. With 200-300% the EDTA-effect was usually apparent within 10-15 min.

Fig. 1-3 illustrate the capillary exchange of THO,  $K^+$  and sucrose or dextran 75 000 during perfusions with plasma containing 120% EDTA. The addition of EDTA to the perfusates lead to a tremendous increase of the permeabilities of  $K^+$  and the other water soluble molecules whereas the permeability of THO continued unchanged. In some experiments the  $P_{THO}/P_{K^+}$  ratio changed from 27 to about 1.3 during EDTA perfusion. This latter value is close to the ratio between the corresponding free diffusion coefficients in water. From the values obtained in 14 expts. with distinct (but not always maximal) EDTA effects the mean  $P_{THO}/P_{K^+}$  ratio was 2.9 when the EDTA-effects were most pronounced (Table II). As for the other watersoluble substances they seemed to follow  $K^+$  very closely. At maximal EDTA effects the mutual relationship between the exchange of water soluble substances was very similar to that between their diffusion coefficients in water (Table II).

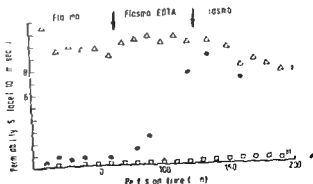


Fig. 2 The effect of EDTA. The onset of the increase in  $PS_{Cl^-}$  was significantly delayed compared to that of  $PS_{K^+}$ .

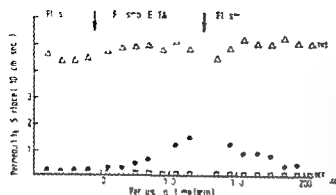


Fig. 3 The effects of EDTA During the perfusion with EDTA (120 of total plasma content of Ca + Mg)  $PS_{K^+}$  but not  $PS_{Dex}$  was increased. Reperfusion with normal plasma lead to an almost complete reversibility.

Sucrose was employed as test-substance in 5 expts. In 3 of these the EDTA-effect developed slowly and the increase in sucrose permeability was delayed as compared to the development in  $K^+$  permeability (Fig. 1). In this phase therefore a molecular size dependant restriction to transcapillary diffusion became temporarily apparent. In the remaining two experiments the EDTA-effect developed more quickly and the permeability curves of  $K^+$  and sucrose were parallel.

The onset of the increase in the capillary exchange to macromolecules (dextran 16 000 and dextran 75 000) was always delayed as compared to the other water-soluble test molecules. Usually the rise in the permeability of these substances could not be recognized until prior to the attainment of maximum permeabilities of smaller water soluble molecules. The *rete* often showed signs of edema as judged from its macroscopical appearance within 1 h after the onset of the rise in dextran permeability. At such circumstances the measurements of the permeabilities becomes inaccurate. As for the lipid-soluble molecules they followed THO very closely. The  $P_{ethanol}/P_{THO}$  and  $P_{anesthetics}/P_{THO}$  ratios remained constant throughout the EDTA perfusions even during the stages in which the *rete* was showing signs of edema and deterioration.

Some of the EDTA treated *retia* were examined by light microscopy and by electron microscopy. No changes in the capillary structure could be observed even in those *retia* showing a significant rise in dextran permeability (Fig. 4).

#### *The reversibility of the EDTA-effect*

In many experiments approximately half of those presented in Table II attempts to reverse the permeability changes induced by EDTA were performed in the following manner. When the EDTA-effect was definitely established as judged from the measurements of  $K^+$  during the experiment normal plasma perfusates were again employed. Since both the time of latency for the occurrence of the initial permeability changes induced by EDTA and also the speed of the permeability changes after this point were highly unpredictable it was difficult to standardize these experiments.

When the EDTA-effect developed very slowly however as was the case in three experiments (120  $\mu$  EDTA) it could be reversed. One of these successful experiments is shown in

TABLE II Mean capillary permeability ratios obtained during maximal effects of EDTA low and high pH

Perfusate	Number of experiments	Initial ratio $P_{TBO}/P_{K^+}$	Mean ratios obtained when the effects were maximal								
			$\frac{P_{TBO}}{P_{K^+}}$	$\frac{P_{KClO_4}}{P_{TBO}}$	$\frac{P_{A. tibialis}}{P_{TBO}}$	$\frac{P_{Mg^{2+}}}{P_{K^+}}$	$\frac{P_{U.a.}}{P_{K^+}}$	$\frac{P_{G. rose}}{P_{K^+}}$	$\frac{P_{O_2 \text{ trans-16}}}{P_{K^+}}$	$\frac{P_{D. lvs. 75}}{P_{K^+}}$	
Plasma + 4-12 mM/l EDTA	14	77	9	103	0.90	0.82	0.83	0.34	0.09	0.05	
Plasma											
pH 3.5-7	4	21	12	0.97				0.41	0.12	0.09	
Plasma											
pH 9.2-10.0	5	32	16		0.81				0.11	0.06	
Corresponding ratios between the free diffusion coefficients in water at 10°C											
Normal permeability ratios of the retial capillaries (Stray Pedersen and Steen 1975)											
			127	0.5	0.8	0.74	0.72	0.77	0.038	0.018	
			36.2	1.02	0.86	0.78	0.94	0.31	—	—	



effect had been partially reversed the  $[K^+]/[Na^+]$  ratio was seen to lie between the plasma value and the normal value of *rete* tissue

#### *Experiments with dialyzed plasma*

From the results obtained in the experiments mentioned above it must be concluded that the presence of  $Ca^{++}$  or  $Mg^{++}$  or both is needed to maintain the permeability properties of the *rete* capillaries. A study of the relative importance of these ions would necessitate perfusions with plasma from which  $Ca^{++}$  or  $Mg^{++}$  had been removed. Such perfusates could be obtained by dialysis of plasma following acidification. The influence of pH on the binding of calcium and magnesium was investigated in a sequence of dialysis experiments. Varying amounts of HCl, NaOH or EDTA were added to plasma and Ringer solutions which were dialyzed against tap water or deionized water. pH and the concentrations of Na, K, Ca and Mg were measured before and after the dialysis. During the dialysis of plasma flocculation of proteins (globulins) would occur. The plasma dialyzate was therefore centrifuged and the analyses were performed in the supernatant. In some instances the content of calcium in the precipitate was also determined. Prior to this the precipitate had been repeatedly washed in deionized water and centrifuged and then dissolved in a Ca- and Mg-free saline solution. Some typical results from these experiments are shown in Table IV.

As expected more than half of the total Ca and Mg was removed when plasma of normal pH was dialyzed against deionized water (Dialysis 1 in the table). Most of the Ca and Mg was found in the supernatant (albumin fraction); almost nothing could be detected in the precipitate (globulin fraction) (Dialysis 2).

To our great surprise an increase of the content of both  $Ca^{++}$  and  $Mg^{++}$  was observed when plasma of normal pH was dialyzed against tap water (Dialysis 6). In spite of a 20-fold higher concentration of Ca and Mg in plasma than in water both substances were absorbed by the plasma, i.e. against the apparent gradient. In the Ringer solutions Ca and Mg existed as free ions only and were hence effectively removed during dialysis against deionized water as well as against tap water (Dialysis 8).

As for the EDTA-containing solutions it should be emphasized that this chelating agent was always added in excess to the content of total calcium and magnesium. However, when added to plasma which was then dialyzed against tap water (Dialysis 7) only about 30% of the calcium and magnesium was lost, i.e. the content of Ca and Mg in the dialyzate was significantly greater than the fraction representing protein-bound Ca and Mg (see Dialysis 1). This finding could not be explained by an interference between the EDTA added and Ca- and Mg-ions outside the dialyzing bag, since similar results were obtained when dialysis was carried out against deionized water (Dialysis 3). As seen from the results obtained with Ringer solutions (Dialyses 8 and 9) a significant amount of Ca and Mg was retained when EDTA had been added (Dialysis 8). It was therefore reasonable to suggest this to be due to permeability properties of the dialyzing bag, the membrane of which must offer considerable restriction to the diffusion of the EDTA-chelate complexes as compared to that of the free ions of Ca and Mg. Unfortunately no information about the pore sizes of the dialyzing membrane employed could be obtained from the producer. At all events, by comparing the results obtained in Dialyses 6, 7 and 9 it must be concluded that EDTA

TABLE IV The content of Na K Ca and Mg in different plasma and Ringer solutions before and after dialysis — = not measured

Performance of the dialysis	Dialysis number	Solutions	pH		Na mEq		K mEq/l		Ca mEq/l		Mg <sup>a</sup> mEq/l	
			Before	After	Before	After	Before	After	Before	After	Before	After
Dialysis against deionized water (6 hrs + 4 C)	1	Plasma normal pH (supernatant)	8.2	7.6	1.8	0.4	3.4	0.2	5.2	2.1 <sup>b</sup>	1.5	0.5
	2	Plasma normal pH (precipitate dissolved in saline)	do	5.0	do	—	do	—	do	0.2 <sup>a</sup>	do	0.1
	3	Plasma + 4 mM/l EDTA (supernatant)	7.8	7.5	—	—	—	—	5.1	3.4	—	—
	4	Plasma low pH (do)	3.7	3.9	—	0.2	3.2	0.1	5.1	0 <sup>b</sup>	1.4	0 <sup>b</sup>
Dialysis against tap water (38 hrs + 4 C)	5	Plasma high pH (do)	9.7	9.5	—	0.2	3.2	0.1	5.2	5.0	1.2	1.1
	6	Plasma normal pH (supernatant)	7.6	7.4	1.2	0.4	3.4	0.1	4.8	5.0	1.4	1.6
	7	Plasma + 4 mM/l EDTA (do)	6.9	7.2	1.27	0.3	3.2	0.1	4.8	5.4	1.3	1.0
	8	Ringer	4.1	5.9	1.40	0.2	4.5	0.1	4.2	0.1	4.5	0.1
	9	Ringer + 4 mM/l EDTA	3.6	6.8	1.41	0.3	4.4	0.2	4.2	1.7	2.3	0.3
	10	Plasma low pH (supernatant)	3.9	4.0	—	0.1	3.3	0.1	4.9	0.1	1.1	0.1
	11	Plasma high pH (do)	9.6	8.7	—	0.4	3.0	0.1	4.8	7.3	1.2	2.0
Deionized water		Tap water	6.2		0.2		0.1		0.2		0.1	
		Deionized water	5.8		—		—		0 <sup>b</sup>		0 <sup>b</sup>	

<sup>a</sup> Measured directly with Atomic absorption photometer<sup>b</sup> Measured with Atomic absorption photometer after evaporation of the sample (see Methods)

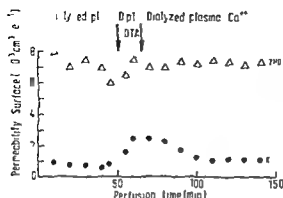


Fig 8 The reversibility of the EDTA effect with  $\text{Ca}^{++}$ . The EDTA-effect developed rapidly when 0.8 mM/l EDTA was added to the dialyzed plasma. A reversibility was obtained during the final perfusion with dialyzed plasma containing 0.8 mM/l EDTA and 2.5 mM/l of  $\text{CaCl}_2$ .

by no means combines quantitatively with the protein bound Ca and Mg in plasma. At this pH therefore the binding of Ca and Mg to the plasma proteins must be stronger than those between Ca/Mg and EDTA. This is rather surprising considering the very low conditional constants of the Ca and Mg EDTA complexes.

When the dialysis was performed at pH values below 4.0 Ca and Mg were effectively removed (Dialyses 4 and 10). When dialyzed against deionized water (Dialysis 4) the concentrations of these substances became unmeasurable ( $[\text{Ca}] < 0.005$  and  $[\text{Mg}] \sim 0.0025$  mM/l). On the other hand a great increase in the Ca/Mg binding capacity of the proteins was observed when the plasma had been alkalinized. During dialysis against tap water at pH of about 9.5  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  were absorbed from the water giving a 50% increase in the content of both substances (Dialysis 11). When deionized water was used less than 10% of the total Ca + Mg was lost (Dialysis 5) whereas more than 50% was lost at normal pH values (Dialysis 1).

#### *Perfusions with dialyzed plasma. Reversibility of the EDTA-effect with $\text{Ca}^{++}$ or Mg*

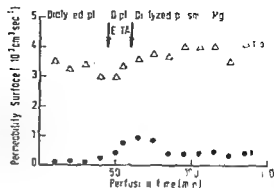
Experiments were performed with plasma which had been dialyzed at pH 3.0–3.5 and to which appropriate amounts of NaCl and KCl had been added (but not  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$ ). Such perfusions could last for 4–5 h. No changes in the permeability properties of the capillaries could be observed.

Several attempts were made to reverse the EDTA induced effects by addition of either Ca or Mg. This was performed as follows. The rete was initially perfused with dialyzed ( $\text{Ca/Mg}$  free) plasma. Then an EDTA-effect was induced by addition of 0.8 mM/l EDTA. The EDTA-effect would usually appear within 10 to 15 minutes. Now the perfusate was exchanged with one of the two following solutions:

1. Dialyzed plasma + 0.8 mM/l EDTA + 2.5 mM/l  $\text{Ca}^{++}$
2. Dialyzed plasma + 0.8 mM/l EDTA + 2.5 mM/l Mg

In 4 expts. only we succeeded in changing the perfusate during the initial phase of the EDTA-effect. In one experiment with each perfusate the EDTA-effect was actually reversed (Fig. 8 and 9). In the two other experiments the introduction of the Ca/Mg-containing per-

Fig 9 The reversibility of the EDTA-effect with  $Mg^{++}$ . The EDTA-effect was elicited by addition of 0.8 mM of EDTA to the dialyzed plasma. A reversibility was obtained with the same type of perfusate to which 2.5 mM of  $MgCl_2$  had been added



fusates happened to occur somewhat later in the development of the EDTA-effect *i.e.* just prior to the increase of the dextran permeability. In these experiments the increase of the permeabilities continued without any delay as was also the case in the other non successful experiments of this type.

### Discussion

The normal permeability relationships of the capillaries in the *rete mirabile* have been presented in an earlier paper (Stray Pedersen and Steen 1975). The capillary membrane was found to be 36 times more permeable to THO than to  $K^+$  (Table II). The mutual relationship between the permeabilities of the smaller water soluble molecules was almost identical to those between the corresponding free diffusion coefficients. The permeability of the lipid soluble molecules were proportional to their lipid solubility. These findings indicated that the capillary membrane has three pathways for transcapillary diffusion. One with lipid character, one allowing water only and one that allows smaller hydrophilic molecules and ions (including water) to pass without progressive size or charge dependent restriction. The intercellular junctions or slits are candidates for this latter pathway. The two first mentioned pathways are most likely situated in the endothelial cell membrane.

The addition of EDTA produced characteristic and reproducible effects on the capillary permeability in the *rete*. Similar responses to EDTA were also obtained in our investigation on the gill membrane (Steen and Stray Pedersen 1975). Possible effects of the decreased pH following the addition of EDTA to the perfusates were excluded by low pH perfusion experiments mimicking the pH effect caused by EDTA. There is a possibility that the permeability changes were caused by chelation of EDTA to other unidentified molecules, but we consider this unlikely. We feel therefore confident that the main effect of adding EDTA is to reduce the concentration of calcium and magnesium ions in the perfusate and in the tissue.

The EDTA-effect consisted of 3 consecutive phases

1. An initial phase in which the capillary permeability to smaller hydrophilic molecules increased dramatically, whereas the permeability to THO and lipid-soluble molecules was not affected.
2. A second stage in which the capillaries became significantly permeable to macromole

cules. The fact that the  $P_{\text{dextran}}/P_{\text{K}^+}$  ratios were higher than the ratios between the corresponding (theoretical) diffusion coefficients in water must be ascribed to inhomogeneities of the dextran fractions employed. The onset of increased dextran permeability seemed to represent the point where the EDTA-effect no longer could be reversed.

### 3. A final phase in which the *rete* showed signs of edema and deterioration

The time needed for these phases to develop depended on the EDTA-concentration and the perfusion rate.

The increase in capillary permeability caused by removal of  $\text{Ca}^{++}$  and/or  $\text{Mg}^{++}$  is generally assumed to be due to a weakening of the cellular adhesions, i.e. making the intercellular slits or junctions to behave as wider channels. Three lines of evidence supports the view that the endothelial cell membrane represents the primary site of action of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ .

1. If the effect of the removal of  $\text{Ca}$  and  $\text{Mg}$  should be a widening of the intercellular slits or an increase in the total slit area to such extent as to give 20-fold increase of  $\text{K}^+$  permeability, then a significant rise in the exchange of THO should be expected. This was not found.

2. A normal *rete* contains almost equimolar amounts of  $\text{K}^+$  and  $\text{Na}^+$  (Stray Pedersen and Nicolaysen 1975) whereas *retia* treated with EDTA showed an ionic composition very similar to that of plasma (Table III). In the experiments in which the EDTA effect was reversed, the  $[\text{K}^+]/[\text{Na}^+]$  ratios were more similar to the values of normal *retia* (Table III).

3. In some experiments (Fig. 1) a transient restriction of sucrose diffusion as compared to  $\text{K}^+$  diffusion was observed during the initial phase of the EDTA-effect. This could indicate a recruitment of new pores which were initially too small to allow the passage of sucrose.

As for the second phase of the EDTA-effect—characterized by an increased capillary permeability to dextrans—this is probably due to changes at the levels of the junctions. However, as seen from Fig. 4 the ultrastructure of dextran permeable capillaries appeared to be normal without widenings of the junctional gaps. The same was also observed in capillaries of the rabbit lung sufficiently exposed to EDTA to give edema (Hovig *et al.* 1971). This does by no means exclude that great changes may have taken place *in vivo*. Earlier studies indicate that the junctional gaps in normal retial capillaries might be considerably wider (average diameter = 1300 Å) than seen on the micrographs (Stray Pedersen and Steen 1975). The fact that these channels offer substantial restriction to the diffusion of high molecular dextrans is most probably due to the presence of a fibrillar matrix or cement substance which functions as a filter. When  $\text{Ca}^{++}$  (and  $\text{Mg}^{++}$ ) is removed this matrix may change in such a way as to create pathways through which the dextrans may pass. The fact that it was possible to reverse the EDTA-effect by addition of equimolar amounts of either  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  to the  $\text{Ca}^{++}/\text{Mg}^{++}$  free plasma containing 0.8 mM EDTA (Figs. 8 and 9) indicated these ions to be of qualitatively and perhaps also of quantitatively equal significance for the maintenance of normal permeability characteristics.

The EDTA-experiments showed that very small amounts of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  are necessary for maintenance of normal permeability characteristics. This is in agreement with results in earlier investigations performed on frog mesenterium (Chambers and Zweifel 1960), frog gastric mucosa (Sedar and Forte 1964) and rabbit lungs (Nicolaysen 1971 a, b). In our experiments the lowest EDTA-concentration which elicited permeability effects was 120

of the total plasma content of  $\text{Ca}^{++}\text{Mg}^{++}$ . This corresponds to  $[\text{Ca}^{++}]$  and  $[\text{Mg}^{++}]$  in plasma of about  $7 \times 10^{-3}$  and  $1 \times 10^{-3}$  mM/l respectively. In the dialyzed plasma which caused no permeability effect  $[\text{Ca}^{++}]$  was less than  $5 \times 10^{-3}$  and  $[\text{Mg}^{++}]$  less than  $2.5 \times 10^{-3}$  mM/l. The "critical" plasma concentrations of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  therefore appears to lie between these values. This is in fair agreement with values found by Nicolaysen (1971 a, b) for the rabbit lung. However, it must be emphasized that these calculations of the concentrations of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  in the presence of EDTA in plasma are based on the assumption that EDTA would combine effectively and equimolarly to all of the Ca and Mg in plasma. According to the results obtained in our dialysis experiments this assumption may be rather doubtful.

In contrast to the EDTA-effect the changes in the capillary permeabilities caused by extreme hydrogen ion concentrations in the perfusates occurred very abruptly. When the pH was brought close to the critical values, only moderate shifts in pH were necessary to elicit the effects, thus indicating an all-or none-effect. For instance at pH 3.9 and 8.8 normal permeability properties were maintained, while at pH 3.5 and 9.4 the effects were dramatic (Fig. 5 and 7). The effects caused by high or low pH could not be reversed. The main pattern of the permeability changes (Table III) was similar to that obtained during EDTA-exposure. This could indicate that the mechanisms of the permeability effects of EDTA and high/low pH are the same. The fact that addition of calcium induced a delay in the response to low pH (Fig. 6) is supporting evidence to this proposal.

We suggest that our findings can be explained in the following way:

Under normal conditions  $\text{Ca}^{++}$  is associated with the cell membrane and exerts its function by its charge, the volume it occupies and possibly by allosteric effects. It may therefore function as a positively charged plug. When the *rete* is exposed to excess EDTA, the  $\text{Ca}^{++}$  ions will leave the membrane and become bound to EDTA. At the same time EDTA will enter the endothelial cells and  $\text{Ca}^{++}$  will leave it. As more and more  $\text{Ca}^{++}$  plugs are removed, the cell membrane loses progressively both its selectivity and its restriction to diffusion. At a certain stage, all  $\text{Ca}^{++}$  will be gone and the cell membrane can be compared to a lipid protein sheath with a certain porosity. Since  $\text{Ca}^{++}$  also has a structure-stabilizing function, the cell to cell adhesion will be destroyed during such conditions. During an early stage, when some  $\text{Ca}^{++}$  is still present, the process is reversible.

We know that at a low pH the affinity of plasma proteins to  $\text{Ca}^{++}$  is lowered, at a high pH it is increased. In artificial (phospholipid) membranes Seimiya and Ohki (1973) have shown the degree of  $\text{Ca}^{++}$  absorption to be highly influenced by pH. It seems reasonable that the proteins (and phospholipids) of the endothelial cell membrane would behave similarly. A low pH will therefore cause release of  $\text{Ca}^{++}$  from the cell membranes, whereas a high pH may strengthen the  $\text{Ca}^{++}$  protein bonds so that  $\text{Ca}^{++}$  can no longer act as a charged plug. Alternatively, high pH may increase the affinity of plasma proteins so that they in fact act as EDTA. The reason why pH-effects give a more rapid effect than EDTA may be that  $\text{H}^{+}$  ions pass the endothelial cell membrane much more rapidly than do  $\text{Ca}^{++}$  or EDTA. In frog skeletal muscle  $\text{H}^{+}$  has been found to diffuse more than 100 times faster than  $\text{K}^{+}$  (Woodbury *et al.* 1968).

Our observations in the dialysis experiments concerning the effects of high and

are in agreement with data reported by Pedersen (1969). He found a linear relationship between the fraction of ultrafiltrable Ca and pH in plasma within a pH range of 6 and 9. Extrapolation from this curve indicates that 100% of the calcium content would be ultrafiltrable at pH 4 and 0% at pH 10. This conforms to the results obtained in Dialyses 4 and 5 in Table IV.

Plasma  $[Ca^{++}]$ ,  $[Mg^{++}]$  or  $[H^+]$  necessary for permeability changes to develop in the present investigation have never been described in systemic blood *in vivo*. Extreme  $[H^+]$  however has been found locally in many organs such as for instance exercising muscles (Barcroft 1968). It has also been postulated that the capillary permeability of skeletal muscles increase during exercise (Bolme and Edwall 1971). The fact that the significant shift in venous pH of working muscles is accompanied by marked changes in  $[Na^+]$  and  $[K^+]$  (Barcroft 1968) is interesting in this connection. On the basis of the present investigation it is tempting to suggest the increase in  $[K^+]$  observed is due to locally decreased pH and thereby removal of  $Ca^{++}$  and  $Mg^{++}$  from the tissue including the capillary membranes. In this way local changes in pH in skeletal muscles and perhaps also in other organs may function to regulate the capillary permeability.

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in human infants (Aperia *et al* 1972, Aperia *et al* in press, McCance and Widdowson 1957) piglets (McCance and Widdowson 1957) rats (Trimble 1970) and dogs (Kleinman and Reuter 1974).

In the present study the natriuretic response to a saline infusion has been determined in lambs of two age groups. The response has been related to developmental changes in intrarenal hemodynamics. The relation of salt excretion to the hemodynamics of the kidney was mainly motivated by the importance of blood flow for the control of intrarenal physical forces under normal physiological conditions partly also by the possible effect of intrarenal blood flow distribution on total salt excretion.

## Material and Methods

**Preparation of animal.** Studies were performed in 13 lambs of both sexes, nine aged 5–8 days and four aged 48–57 days. All lambs were given possibility of sucking or free fluid intake up to about 2 h before the experiment, each of which was performed on approximately the same time of the day. The animals were anesthetized with penthotal sodium (0.25 mg/kg b.wt.), intubated for ventilation (oxygen and nitrous oxide) with an Engstrom respirator and were given atropine (0.05 mg/kg b.wt.) and pancuronium bromide (Pavulon 0.02–0.03 mg/kg b.wt.) for muscle relaxation. They were placed on a heat controlled table to maintain rectal temperature at 37.5–38.0°C.

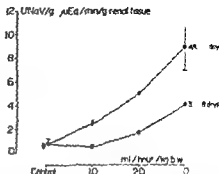
Cannulas were inserted into one brachial and one femoral vein for infusions. Both femoral arteries were cannulated with an end hole polyethylene catheter. One was introduced into the left ventricle for blood pressure recordings and for injections of microspheres and Cardio-green. The position of the catheter tip in the left ventricle was verified by the shape of the pressure curve. Blood samples were drawn from the other femoral artery. Both ureters were dissected after a suprapubic incision and cannulated (infant feeding tube no. 5) for the purpose of urine collection.

**Experimental protocol.** For the determinations of glomerular filtration rate (GFR) and urinary sodium excretion ( $U_{Na}V$ ) standard clearance techniques were used. The studies were performed during the continuous infusion of inulin ( $10^{-4}$  Laevosan Ges. Linz, Austria) (0.15 ml/min/kg b.wt.). The infusion was started 60 min before the first urine collection period and preceded by a prime dose of 0.5 ml/kg b.wt. Urine was collected during 15 min periods and arterial blood samples were taken at the mid point of each collection period. Following two control periods an intravenous infusion of a saline solution (Na 147 mEq/l) was added. During the first 45 min experimental period it was administered at a rate of 10 ml/h/kg b.wt. The infusion rate during the following two periods was 10 and 30 ml/h/kg b.wt. respectively. At the end of each experiment (45 min) a total saline volume of 0.75, 5\* and 4.5 of b.wt. respectively had then been injected. Calculations from urine collected during the last 15 min of each period was registered. Blood flow determinations were performed immediately before and after the progressive isotonic ECV expansion.

**Hemodynamic studies.** Distribution of renal cortical blood flow before and after volume expansion was determined with radioactive microspheres (3M Company, St. Paul, Minnesota, USA) labelled with different isotopes ( $Cr^{51}$  and  $Ce^{141}$  respectively). The microspheres measuring 15  $\mu$ m in diameter were suspended in 10% dextran and a few droplets of Tween 80 as a detergent. A sample of 1 ml (including about 400 000 microspheres) was treated with ultrasonic agitation to a solid adherent and then injected into the left ventricle during 5–10 s. In order to determine absolute tissue flow the cardiotemp was recorded with a dyed dilution technique (Cardiogreen) previously described (Aperia *et al* 1974). Blood pressure was recorded on a Grass polygraph with a Statham pressure transducer.

**Anatomy of tissue.** Just before sacrifice the animals had a ink (up to 0.1 ml) was injected into the aorta just above the entrance of the renal arteries. The ink was given for marking the glomeruli to facilitate cortical mapping. The kidneys were then removed, weighed and cut longitudinally. Three to four about 1 mm thick sagittal renal tissue blocks were sectioned. The cortex was cut out of each block with a thin and sharp knife under a dissecting microscope into three circumferential pieces—an outer, a middle and an inner cortical layer each occupying about 10, 65 and 25% respectively of the total cortical volume. The inner cortical slices corresponded approximately to the zone where the majority of the juxtaglomerular nephrons are situated. Each cortical slice was weighed wet and counted in a Packard Auto-gamma spectrometer.

Fig. 1 Mean urinary sodium excretion per gram renal tissue ( $\mu\text{Eq min g}$ ) ( $\pm \text{S.E.}$ ) during isotonic saline infusion with three successively increasing infusion rates. The recordings were made when each infusion rate had been maintained for 45 min.



**Analytical methods** The chemical analyses of insulin in serum and urine were performed according to the Anthron method described by Liljeberg *et al.* (1958). Serum total protein concentration was analyzed with a refractometric method. Sodium was determined with a flame photometer (Eppendorf).

**Calculations** The regional cortical tissue flow was counted from the formula  $q = \text{CO} (Q - w)$  where  $q$  and  $Q$  is the count rate in the individual slices and that injected into the animals respectively. CO is the cardiac output and  $w$  is the weight of the individual slices.

## Results

**Urinary sodium excretion** The saline infusion resulted in a continuous increase of urinary sodium excretion in all animals (Fig. 1). The increase was out of proportion to the change of GFR (Fig. 1 a, b). There was, however, a difference in the degree of response in the two groups studied. In the younger lambs the natriuresis was much less marked at each level of ECV-expansion.

**General effects of ECV-expansion** There was no apparent effect of ECV-expansion on cardiac output or left ventricular systolic blood pressure in any of the animals studied (Fig.

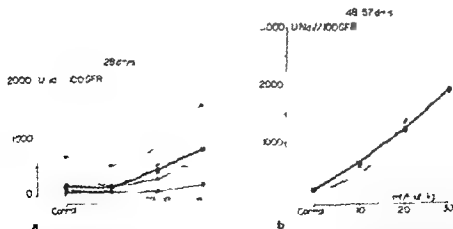


Fig. 2 a, b Urinary sodium excretion ( $\mu\text{Eq min}$ ) per unit filtered load during isotonic saline infusion with three successively increasing infusion rates. The small filled circles connected with thin lines represent the individual recordings in the two age groups (5-8 days (Fig. a) and 48-57 days (Fig. b)). The large filled circles connected with heavy lines represent the corresponding mean values.

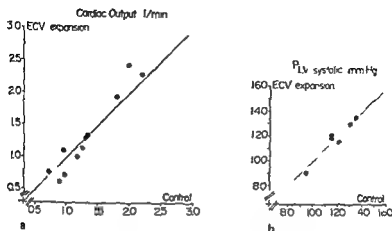


Fig 3a b Effects of saline infusion (4.5% of b wt) on cardiac output (Fig 3a) and left ventricular systolic blood pressure (Fig 3b) Line = one of identity

3 a b) Nor was there any effect on the serum sodium value (Fig 4) The ECV-expansion resulted in a significant fall ( $p < 0.05$ ) in serum protein concentration in both age groups (Fig 5) The average fall in serum protein concentration was somewhat larger in the younger animals but the difference between the two age groups was not significant The fall in serum protein concentration was paralleled by a fall in hematocrit (Fig 6)

**Renal hemodynamics** The ECV-expansion had different effects on renal hemodynamics in the two age groups studied As shown in Fig 7 a b the blood flow to the outer and mid cortex did not change much in the 5-28 day old lambs (Fig 7 a) while the blood flow to the inner cortex increased somewhat (Fig 7 b) In the 48-57 day old lambs there was an increase in blood flow to all cortical layers (Fig 7 a b) The relationship between inner and outer cortical blood flow increased somewhat more in the younger lambs than in the older lambs (Fig 8)

**Glomerular filtration rate** The values for glomerular filtration rate (GFR) found during the control periods were in accordance with those previously recorded in this laboratory in

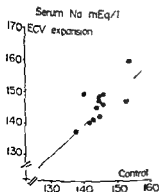


Fig 4 Effect of saline infusion (4.5% of b wt) on serum sodium concentration Line is one of identity

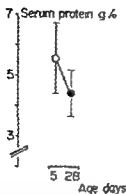


Fig. 5

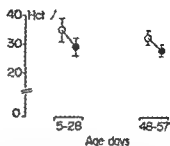


Fig. 6

Fig. 5 Effect of saline infusion (4.5% of b.wt.) on mean serum protein concentration in the two age groups. Unfilled circles represent control values ( $\pm$  S.D.) filled circles represent values when saline had been infused ( $\pm$  S.D.)

Fig. 6 Effect of saline infusion (4.5% of b.wt.) on mean hematocrit in the two age groups. Unfilled circles represent control values ( $\pm$  S.D.) filled circles represent values when saline had been infused ( $\pm$  S.D.)

developing lambs (Aperia *et al.* 1974). Thus there was an exponential increase in glomerular filtration rate per gram renal tissue with increasing age. Fig. 9 demonstrates a comparison between the GFR before and after ECV-expansion in each animal studied. The degree of volume expansion used had little effect on the GFR in either age group.

It thus appears as if there is a discrepancy between the effect of ECV-expansion on renal blood flow and on glomerular filtration rate in at least the older animals. This discrepancy is confirmed in Fig. 10 where the relationship between GFR and cortical blood flow has been examined in both age groups studied. In the younger animals there was no significant

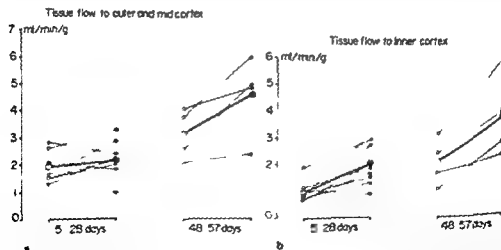


Fig. 7a, b. Individual changes of blood flow per gram renal tissue (ml/min/g) to outer and mid cortex (Fig. 7a) and to inner cortex (Fig. 7b) in the two age groups before and after saline infusion (4.5% of b.wt.). The small unfilled circles represent control values, the small filled circles represent flow values when saline had been infused, the corresponding large circles represent mean flow values respectively.

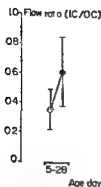


Fig 8

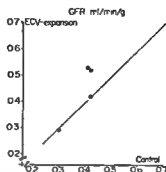


Fig 9

Fig 8 The inner to outer mean cortical blood flow ratio in the two age groups before and after saline infusion (4.5 of b wt.) Unfilled circles represent control values ( $\pm$  S.D.) filled circles represent the flow ratio when saline had been infused ( $\pm$  S.D.)

Fig 9 Effect of saline infusion (4.5 of b wt.) on glomerular filtration rate per gram renal tissue (GFR/g). Line is one of identity

difference between the GFR to cortical blood flow quotient before and after expansion while in the older animals there was a significant decrease ( $p < 0.05$ ) in the quotient during ECV-expansion. Since the effect of ECV-expansion on hematocrit was similar in the two age groups it can be concluded that the GFR to plasma flow ratio i.e. the filtration fraction was more reduced in the older lambs.

### Discussion

The use of the microsphere method for intrarenal blood flow determinations has been evaluated previously (Katz *et al* 1971 Slotkoff *et al* 1971). The method has been found to be reliable under standardized conditions. The microspheres are supposed to mainly indicate the distribution of red cells that are subject to axial streaming. It might thus be influenced by changes in hematocrit. Therefore the reliability of the method for repeated determinations during ECV-expansion has been discussed (Blantz *et al* 1971 Wallin *et al* 1971). The main purpose of this study however has been to relate the intrarenal tissue flow before and after volume expansion to age. The possible error of the microsphere method should therefore influence the results to the same extent in the two age groups.

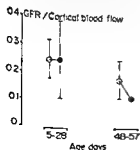


Fig 10 The quotient between glomerular filtration rate (GFR) and cortical blood flow in the two age groups before and after saline infusion (4.5 of b wt.). Unfilled circles represent control values ( $\pm$  S.D.) filled circles represent the ratio when saline had been infused ( $\pm$  S.D.)

A number of recent studies have demonstrated that the response to a salt and fluid load is suppressed in several newborn species (Aperia *et al* 1972 Aperia *et al* in press Kleinman and Reuter 1974 McCance and Widdowson 1957 Trimble 1970). In the present study it was found that the renal response to an isotonic ECV-expansion is age dependent also in lambs. The urinary sodium excretion per unit filtered load was markedly lower in the 5-28 day old lambs than in the 48-57 day old lambs.

Changes in intrarenal physical forces that will result in inhibition of proximal tubular reabsorption of sodium are thought to be an important mechanism for the natriuretic response to an ECV-expansion in adult animals (Brenner *et al* 1971 Grandchamp and Boulpaep 1974 Martino and Earley 1967 Windhager *et al* 1969). These forces are governed by the transtubular oncotic and hydrostatic pressure gradients. The changes of the gradients are mainly determined by the oncotic and hydrostatic pressures in the peritubular capillaries. The oncotic pressure is dependent on the serum protein concentration and on the filtration fraction. The hydrostatic pressure in the peritubular capillaries is dependent on the arterial blood pressure and on the relationship between the resistance in the afferent and the efferent arterioles. It is thus apparent that changes in intrarenal hemodynamics will have great consequences for the reabsorption of sodium and water by exerting an effect on intrarenal physical forces.

In the present study the following parameters that could influence intrarenal physical forces have been followed: the serum protein concentration, the filtration fraction and the arterial blood pressure. The serum protein concentration fell markedly in both age groups. In the younger animals there was a slight and insignificant increase in both RFR and total cortical blood flow. In the older animals there was also a slight increase in GFR while there was a fairly pronounced increase in the tissue flow to all cortical layers following the ECV-expansion. This resulted in a significant fall in the GFR to cortical blood flow quotient. During ECV-expansion the hematocrit decreased to about the same extent in all animals (Fig. 6). The decrease in the filtration fraction must therefore have been more pronounced in the oldest age group. The fall in serum protein concentration was equivalent in the two age groups. It can therefore be concluded that the serum protein concentration and thus the oncotic pressure in the peritubular capillaries was more reduced in the older animals than in the younger animals following the expansion. This difference might at least in part explain the difference in fractional salt excretion in the two age groups of animals. No change was recorded in the arterial blood pressure during the ECV-expansion. It can, however, not be excluded that the expansion resulted in changes in intrarenal hydrostatic pressure. The change in filtration fraction induced in the older animals suggests that the relationship between the vascular resistance in the afferent and in the efferent arterioles was also changed. The fact that the renal blood flow increased while the cardiac output remained fairly constant during the expansion also indicates that the expansion induced some specific changes in the renal vascular system.

Although the importance of physical forces is well established it has been suggested that other factors will also contribute to the natriuretic response induced by an ECV-expansion. One of those is a functional redistribution of the filtrate (Barger 1966 Earley and Friedler 1965). This would mean that the proportion of the filtration occurring in the superficial

nephrons will increase in comparison to the filtration in the deep nephrons. Since it is suggested that the short proximal tubules of the outer cortical nephrons will reabsorb less sodium than the long proximal tubules of the juxtamedullary nephrons such a redistribution to the superficial nephrons will result in a natriuresis (Horster and Thum 1968, Jansson and Lacy 1971). In the present study the ECV-expansion produced a relative increase in blood flow to the inner cortex in the very young lambs. The functional significance of an intrarenal blood flow redistribution is however unclear (Barratt *et al* 1973, Stein *et al* 1971). There are today no uniform data stating a constant relationship between GFR and glomerular blood flow in the juxtamedullary nephrons. In fact it has been shown with the use of the ferrocyanide technique that in chronically salt loaded rats there is a functional redistribution of the filtrate to the outer cortex that is more pronounced in the younger animals (Baines 1973). The intrarenal blood flow distribution before and after ECV-expansion has previously been studied in 1-30 day old puppies (Kleinman and Reuter 1974). Since however puppies might be in an earlier stage of renal development than lambs of corresponding age (Aperia *et al* 1974) and also because of different cortical sectioning the results from that study (Kleinman and Reuter 1974) cannot be directly compared with the present study.

The present results have not unequivocally pointed to the factor responsible for the suppressed natriuretic response to ECV-expansion in the very young animals. It is quite likely that the inability to decrease the filtration fraction in the youngest animals contributed to the low salt excretion. There are however several factors that are not directly dependent on renal hemodynamics that might also be responsible for the poor natriuretic response to an ECV-expansion. Such factors as the renin-angiotensin system, the proximal enhanced distal tubular sodium reabsorption have however not been evaluated in the present study which primarily aimed at clarifying the role of intrarenal hemodynamics for the control of salt excretion during development.

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## On the Noradrenaline Loading in Axonal Amine Storage Granules in Rat Crushed Sciatic Nerves<sup>1</sup>

By

A. DAHLSTRÖM, J. HAGGENDAL and P. A. LARSSON

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### Abstract

DAHLSTRÖM, A., J. HAGGENDAL and P. A. LARSSON. On the noradrenaline loading in axonal amine storage granules in rat crushed sciatic nerves. *Acta physiol scand.* 1975 94 451-458

The increase of NA in rat sciatic nerve above a crush was investigated. The transported amounts of NA do not increase quite linearly with time but more NA was found above the crush at 6 and 12 h than would be expected from the 3 h value. One possible reason for this phenomenon—an increased NA load on the accumulated axonal granules—was investigated by 2 types of double crush experiments. One type involved simultaneous double crushes 1-1.5 cm apart. The increase in NA in the isolated segment 6 h after crush indicated that the axonal amine storage granules had increased their NA load by about 75%. In the second type ("delayed double crushes") a distal crush was made 6 h before a second crush, 1-1.5 cm proximal to the first crush. 1-1.5 h after the second high crush the NA content of the isolated segment was assayed. The results indicated an increased NA content in the axonal granules of 75% after 3-4 h for the isolation of the segment, remaining constant up to 9 h after the second crush. The results indicate that axonal storage granules may increase their NA content by a factor of about 1.75 while being transported distally in the axons. This information together with the information from the preceding article of a mobile NA fraction of 45% was used to calculate the rate of transport of NA proximal to a crush. The value obtained was 9 mm/h which is in good agreement with the value obtained for transport distal to a crush (8 mm/h) in the preceding article.

The amine storage granules in peripheral adrenergic neurons are synthesized in the perikarya and transported distally in the axons to the nerve terminals (cf Dahlström and Haggendal 1966, Dahlström 1971). The granules are able to store the noradrenaline (NA) in an early stage and contain the NA during their axonal transport. Therefore if a crush is made to a nerve which carries adrenergic axons NA accumulates at a rapid rate proximal to a crush (Dahlström and Fuxe 1964, Dahlström 1965, Dahlström and Haggendal 1966). The protein components of the granules dopamine  $\beta$ -hydroxylase (D $\beta$ H) and chromogranin B also accumulate after crushing (Laduron and Belpaire 1968, Livett,

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### Abstract

DAHLSTRÖM A, J HÄGGENDAL and P A LARSSON. On the noradrenaline loading in axonal amine storage granules in rat crushed sciatic nerves. *Acta physiol scand* 1975 94 451-458.

The increase of NA in rat sciatic nerve above a crush was investigated. The transported amounts of NA did not increase quite linearly with time but more NA was found above the crush at 6 and 12 h than would be expected from the 3 h value. One possible reason for this phenomenon—an increased NA loading of the accumulated axonal granules—was investigated by 2 types of double crush experiments. One type involved simultaneous double crushes 1-1.5 cm apart. The increase in NA in the isolated segment 6 h after crushing indicated that the axonal amine storage granules had increased their NA load by about 70%. In the second type (delayed double crushes) a distal crush was made 6 h before a second crush 1-1.5 cm proximal to the first crush. 1-12 h after the second high crush the NA content of the isolated segment was assayed. The results indicated an increased NA content in the axonal granules of 75% already 3-6 h after the isolation of the segment, remaining constant up to 9 h after the second crush. The results indicate that axonal storage granules may increase their NA content by a factor of about 2 (1.7) while being transported distally in the axons. This information together with the information from the preceding article of a mobile NA fraction of 45% was used to calculate the rate of transport of NA granules proximal to a crush. The value obtained was 9 mm/h which is in good agreement with the value obtained for transport distal to a crush (8 mm/h) in the preceding article.

The amine storage granules in peripheral adrenergic neurons are synthesized in the perikarya and transported distally in the axons to the nerve terminals (cf Dahlström and Häggendal 1966, Dahlström 1971). The granules are able to store the noradrenaline (NA) at an early stage and contain the NA during their axonal transport. Therefore if a crush is applied to a nerve which carries adrenergic axons, NA accumulates at a rapid rate proximal to a crush (Dahlström and Fuxe 1964, Dahlström 1965, Dahlström and Häggendal 1966). The protein components of the granules, dopamin- $\beta$  hydroxylase (D $\beta$ H) and chromogranin II, also accumulate after crushing (Laduron and Belpaire 1968, Livett,

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Gelfen and Rush 1968 Brimjoin 1972 Wooten and Coyle 1973) In parallel also dense core vesicles (presumably identical to amine storage granules) pile up in front of the constriction (Kapeller and Mayor 1967 Gelfen and Ostberg 1969 Dahlström and Hökfelt 1971) Since dense core vesicles have been observed in perikarya and also in uncrushed axons (Hökfelt 1969 Gelfen and Ostberg 1969) it is very likely that these storage organelles are—at least to a large extent—preformed in the cell body and transported distally in the axon as intact organelles

NA or DSH in the axon have been used as markers for amine granules The amounts of the two substances that accumulate above a crush during a certain period of time have been used to calculate the rate of transport of the amine granules When the NA accumulation was used for this purpose (Dahlström and Häggendal 1966) the rate of transport in rat sciatic nerve was calculated to be in the order of 5 mm/h For this calculation a number of assumptions were made 1) All the NA in the sciatic nerve is transported distally in the axons at a fast rate 2) The number of granules that are transported distally in the axons is constant with time at least up to 12 h and 3) The granules contain a constant amount of stored NA—the granules do not increase their stores of NA while accumulated

These assumptions have been reconsidered one by one during the last couple of years In the preceding article (Häggendal *et al.* 1975) assumption no 1 was studied and found to be incorrect only about 50% of the NA in the sciatic nerve is mobile Also assumption no 2 may under certain conditions be non valid the production and distal transport of granules is probably constant for up to 6 h but thereafter in a cold environment the amounts of granules may increase (Dahlström and Häggendal 1972) The last assumption finally is dealt with in the present article 3 types of experiments have been performed At first a careful study was made to investigate whether or not in rat sciatic nerve the increase in NA content above a crush was unquestionably linear 3, 6 and 12 h after crushing Secondly simultaneous double crushes 1.5 cm apart were made and the NA content in the separated nerve segment was measured between 0 and 9 h to see if the NA content was constant or increasing with time after separation Thirdly delayed double crushes were performed In these experiments granules were allowed to accumulate for 6 h above a crush and then a second crush was made allowing the accumulated population of granules to alter their NA content uninfluenced by newly arriving granules The results indicate that axonal amine storage granules do increase their load of NA by approximately 100% during accumulation and probably transport

### Material and Methods

Male rats of the Sprague-Dawley strain (about 200 g) were used and kept at a room temperature of  $23^{\circ}\text{C}$  ( $\pm 1^{\circ}\text{C}$ ) during the whole experiment The sciatic nerves were operated bilaterally under ether anaesthesia with 1 or 2 crushes *cf.* Loh & La (1959)

In the first experiment a single crush was made 0, 3, 6 or 12 h before sacrifice The 0.5 cm or 1 cm of nerve just proximal to the crush was dissected out and assayed for NA content according to Häggendal (1963) Two to 6 nerve segments were pooled for each estimation

In the second experiment 1 s simultaneous double crushes were made at the same time 1.0–1.5 cm apart After 0, 6 or 9 h the rats were killed by a blow on the head and the nerve segment between the crushes was dissected out Particular care was taken that no material from the proximal

side of the high crush or distal to the low crush was contaminating the middle segment 4 to 8 nerves were pooled for estimation.

The third experiment involved delayed double crushes i.e. the 2 crushes were made at different times. The distal crush was always performed 6 h before the proximal crush. Zero 1 3 4 6 9 and 11 h after the second high crush the rats were killed and the segment between the crushes was dissected out. Generally 6 nerves were pooled for the NA estimation.

In every experimental series uncrushed nerves were included pooled in a total length of 10.0 cm and assayed for NA content. Also 100 ng of NA was added to one or more samples to test the recovery of the assay procedure (Haggendal 1963). The recovery was 83% as a mean and was corrected for.

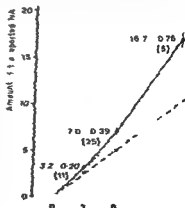
## Results

The results from the first experiment are shown in Fig. 1. The amount of NA which had been transported to the part of nerve just above the crush is indicated (solid line Fig. 1). The transported amount was calculated by subtracting the NA amount in uncrushed or 3 h crushed nerve from the total amount of NA in the crushed nerve. It can be seen (Fig. 1) that the increase in transported NA is not linear. The observed 12 h value is higher than would be expected from the 3 h value (dotted line Fig. 1).

In the simultaneous double crush experiments  $2.21 \pm 0.111$  ng ( $n=16$ ) of NA was present per cm in the separated nerve segment in 0 h crushed nerves. At 6 h after crush the NA content per cm in this isolated segment had increased to  $3.09 \pm 0.215$  ng ( $n=16$ ). As demonstrated in the previous paper (Haggendal *et al.* 1975) about 45% of the NA content in uncrushed nerves is rapidly transportable and probably located within axonal storage granules in this case 45% of  $2.21$  ng =  $0.995$  ng. The rest 55% ( $1.216$  ng) is presumably localized in nerve terminal granules of adrenergic nerve terminals innervating the blood vessels of the sciatic. It is known that nerve terminals increase their NA content by about 15% during the first 6 h after denervation (cf. Benmiloud and Euler 1963; Bareggi *et al.* 1975). Since our double crush procedure caused a denervation of the isolated segment part of the increase observed was presumably confined to the nerve terminal portion of the sciatic NA. The increase in nerve terminal NA during the 6 h of crushing was thus

Fig. 1. The increase in noradrenaline (NA) in rat sciatic nerve above a crush is indicated (solid line). 3 or 10 mm segments just caudal to the crush were dissected out and assayed. The figures for NA increase 3 or 10 h after crushing were obtained by subtracting the NA content of 3 or 10 mm uncrushed nerve from the total NA content of the accumulated segment. Means  $\pm$  S.E. are given. Small numerals indicate number of observations. The dotted line indicates the theoretical NA increase with time had the increase proceeded with the 3 h rate per 3 h as during the first 3 h period.

The hatched line shows the theoretical increase expected to be obtained in case the transported NA granules had not increased the NA loading due to transport and accumulation on (correlation coefficient 0.98) for further explanation see text.



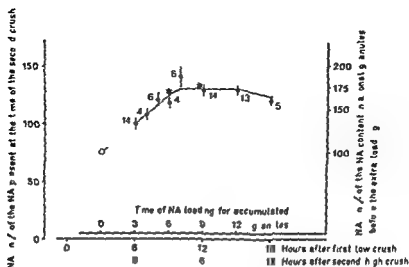


Fig. 1. The increase in noradrenaline (NA) loading in isolated axonal granules of rat sciatic nerve. Axonal storage granules were allowed to accumulate above a low initial crush for 6 h. At this time a second high crush was performed 1.5 cm proximal to the first crush. The second crush prevented a further transport into the isolated segment of axonal granules from more proximal parts of the nerve. 1, 3, 4, 6, 9 and 12 h after the second crush the segment was dissected out and assayed for NA content. The dots (•) represent mean  $\pm$  S.E. (vertical bars) of the NA increase which can be attributed to increase in the NA loading of axonal granules after corrections for the nerve terminal NA (see text). Small figures represent number of observations.

The hatched line indicates an extrapolation to 0 time of the extra loading of NA in the accumulated axonal granules.

The two asterisks indicate the NA increase in axonal granules in the simultaneous double crush experiment.

15% of 1.216 ng = 0.182 ng. Of the total increase of 0.88 ng/cm only 0.70 ng (0.88 - 0.182 ng) therefore appeared to be connected with axonal storage granules. The increase in axonal storage granules was thus from 100% (45%  $\pm$  2.21 - 0.99) to about 170% (0.99 - 0.70 ng). When the 9 h values were used for similar calculations an increase of 173% was obtained.

The results from the delayed double crush experiment are shown in Fig. 2. The stationary NA fraction (55% of the total NA in 11 h crushed nerves) was subtracted from the amount of NA present 8 h after the first crush, i.e. at the time of the second high crush. The values at 1, 2, 3, 4, 6, 9 and 12 h after the second crush were likewise produced by subtracting the stationary NA in the nerve terminals (15% increase after denervation) from the assayed amount of NA in the separated segment between the two crushes. It is clear from Fig. 2 that the amount of NA in the axons of the separated nerve segment increased during the first 3-4 h after the second crush and then remained approximately stable during the next 8-9 h.

### Discussion

As shown in Fig. 1 (solid line) the increase in NA proximal to a crush did not proceed absolutely linearly with time. The increase per unit of time was larger at 1 to 9 and 9 to

1 h than at 0 to 3 h post crushing (compare solid and dotted lines). In the original paper (Dahl's own and Häggendal 1966) the increase was described as approximately linear both in cat and rat sciatic nerves. Later investigators have found the increase to be linear in the 2.5 mm segment above crush in rat sciatic nerve (McLean and Keen 1972) and in the cat hypogastric nerve (Banks and Mayor 1972). The reason why these authors obtained a linear NA accumulation may be that the nerve segment was too short to include the whole extent of the NA accumulation (2.5 mm McLean and Keen 1972) or that the number of observations was too small (Banks and Mayor 1972) to disclose the rather small deviation from the straight line (Fig. 1).

The reason for this deviation from the linearity which implies that more NA than expected was found in the segment proximal to the crush could be either a) that an increase (with time) number of storage granules were transported distally in the axons and/or b) that the accumulated granules increased their NA content. Alternative a) is unlikely because the temperature of the surrounding, which, if low can influence the synthesis of granules in the perikarya (Dahlström and Häggendal 1972) was kept constant at 23°C throughout the experiments. Alternative b) is more likely because it is known that accumulated granules do synthesize NA (McLean and Keen 1972).

In order to examine further if accumulated granules can increase their NA load the unilateral double crush experiment was done. All the accumulated NA in double crushed nerves appears to be reserpine depletable (Dahlström 1967) the NA found in the segment 0 or 6 h after crushing is therefore most probably localized to storage granules. Thus, since the isolated nerve segment contained a fixed number of axonal granules (no supply from more proximal segments because of the high crush) any increase in NA in this segment could be attributed to an increased NA content of the amine granules present in the segment. The NA in the isolated segment increased from 2.21 ng/cm at 0 h to 3.09 ng/cm at 6 h. Only 45% of the 0 h value (2.21 ng) was attributable to axonal storage granules (see Häggendal *et al.* 1975) i.e. 0.995 ng. The nerve terminal granules (containing 1.22 ng of NA) probably increased their NA content by about 15% after the denervation (cf. Benmiloud and Euler 1963; Bareggi *et al.* 1974) caused by the double crush procedure. Therefore about 0.18 ng of the total increase in the isolated segment 6 h after operation was presumably confined to nerve terminal granules along blood vessels in the perineural connective tissue. Thus 0.70 ng of the total increase of 0.88 ng presumably occurred within accumulated axonal granules. These granules consequently appear to have increased their NA content from 0.99 ng to about 1.69 ng, i.e. by about 70%.

The delayed double crush experiments were performed to investigate if a larger amount of accumulated axonal granules (6 h of accumulation before interrupting further supply of granules by the second high crush) with higher initial content of NA would yield a more reliable result in terms of a clear increase. As seen from Fig. 2 the NA content in axonal granules in the isolated segment (total value corrected for nerve terminal NA at 0 h plus the denervation increase in nerve terminals at 1–12 h after the second crush) increased up to 3 h after the second crush and then remained rather constant up to 6 h. At the time of the 2nd crush the axonal granules in the isolated segment had spent on the average 3 h in the segment, since some were arrested there just after the first crush, and other just



arrived to the segment 6 h later at the time of the second crush. The NA curve in Fig. 1 was therefore extrapolated to 3 h before the second crush. This point was considered to represent the NA amount in axonal granules before the onset of the extra loading (100% ordinate to the right in Fig. 2). The increase of NA in these granules after the extra loading would then be about 75%. The asterisks in Fig. 2 represent the corresponding values from the simultaneous double crush experiments. The increased NA loading in the axonal granules in the two experiments was thus very similar, 75% and 70%, respectively. It is interesting that a similar increase in NA appears to occur in bovine splenic nerve granules during transport from proximal to distal segments (1.7 fold increase in the NA/protein ratio Lagercrantz 1974).

These figures (about 75% increased NA content in accumulated axonal granules) may be used to calculate the theoretical accumulation curve for NA above a crush which should be observed were the NA content of the granules constant. This curve is indicated in Fig. 1 (hatched line) and was produced in the following way. Granules which were present above a 3 h crush had been accumulated for 1.5 h as a mean and had increased their NA content to about 117% (corresponding to 3.2 ng) from 100% (2.75 ng). Likewise the 6 h value was corrected for an increase to 133% (7.0 ng) from 100% (5.26 ng). The 12 h value (16.7 ng) was corrected in the following way. If the number of granules which are transported to the segment during 12 h contain  $x$  ng of NA (indicating the relative number of granules) before the increased loading, then  $0.5x$  granules have been present above the crush for 6 to 12 h. These granules have been maximally loaded from 100% to 175% (Fig. 2). The rest of the granules ( $0.5x$ ) have been present in the segment for 0 to 6 h (i.e. 3 h as a mean) and have increased their NA from 100% to 133% (Fig. 2). Thus the total number of granules after the extra loading contained  $0.5x$  (175/100) +  $0.5x$  (133/100) = 16.7 ng  $\approx$  10.8 ng which represents the original NA amount in the downtransported granules before the extra loading. The theoretical accumulation curve (hatched line Fig. 1) obtained in this way is clearly different from the empirical curve (solid line Fig. 1). According to the theoretical curve about 0.90 ng of NA would have been transported towards the crush region per hour. Since the transportable NA fraction in 1 cm of uncrushed nerve (no extra loading) contains 0.99 ng of NA, this constructed accumulation curve indicates a transport rate of about 9 mm/h. This is close to the figure for transport rate obtained in the previous paper on transport distal to a crush (about 8 mm/h Häggendal *et al.* 1974). Also 8 mm/h was found to be the rate of transport for a factor maintaining the nerve terminals (Häggendal *et al.* 1974; Bareggi *et al.* 1974).

When the D/H accumulation above a crush was used to calculate the rate of transport of amine granules in rat sciatic nerve figures close to 8 mm/h were obtained (6.77 mm/h Wooten and Coyle 1973; 9 mm/h Häggendal unpublished).

The increase in NA loading of the axonal amine granules found in this study probably represents an increase occurring in the granules during their normal transport distally in the axons. Their content of D/H is likely to remain constant during transport through the axons. Since the ratio of NA/D/H was found to be higher in distal segments of the sciatic nerve than in proximal parts (Häggendal unpublished) this indicates an increased NA loading per granule during transport (see also Lagercrantz 1974).

3-4 h after the second crush the NA increase in the isolated segment levelled off (Fig. 2) the NA content remaining rather constant up to 1-2 h after crush. This may indicate that the granules have reached their maximal load by 3-4 h. This is not in agreement with suggestions by other authors (*cf.* Stjärne 1968, Lagercrantz 1971) who propose that nerve trunk vesicles (axonal granules) when reaching the nerve terminals may have increased their NA content by several orders of magnitude. These suggestions are based on the large difference in NA/DβH ratio between axonal granules and nerve terminal granules. However, since DβH is lost from nerve terminal granules on repeated transmitter release while NA is resynthesized, the majority of nerve terminal granules have a high NA/DβH ratio due to a loss of DβH—not necessarily due to a largely increased NA loading if compared to axonal granules. The axonal granules have not yet been exposed to DβH loss at transmitter release and therefore contain less NA when related to DβH. Stjärne (1968) has suggested that axonal granules increase their NA content more than 100 times when arriving in the nerve terminals on the basis of the NA/DβH ratio in the 2 populations of granules. We conclude from the present results that axonal NA granules during their transport in the axons may increase their NA load (content) by a factor of 2 (see also Lagercrantz 1974). A further increased NA content per granule in the nerve terminals may be quite possible but it seems unlikely that each granule increases its NA content by 100 times.

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## Synthesis of Catecholamines in the Eye after Local Injection of $^3\text{H}$ -Precursors

By

LEIF RENTZHOOG

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### Abstract

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RENTZHOOG L. *Synthesis of catecholamines in the eye after local injection of  $^3\text{H}$  precursors*  
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The possibility that adrenaline may be synthesized locally in the eye was investigated in the rabbit and vervet monkey. L-tyrosine-3,5- $^3\text{H}$  (L-tyrosine (side-chain 2,3- $^3\text{H}$ )) or L-3,4-dihydroxyphenylalanine ( $^3\text{H}(\text{G})$ ) was injected into the anterior chamber or the vitreous. The animals were killed after 1/2 h up to 7 days in the tyrosine experiments and after 1 up to 4 h in the DOPA experiments. The synthesis of  $^3\text{H}$  adrenaline,  $^3\text{H}$  noradrenaline and  $^3\text{H}$ -dopamine was measured with a modified version of a double isotope technique for determination of catecholamines. No  $^3\text{H}$  adrenaline was found. An attempt to stimulate the phenylethanolamine N-methyltransferase activity with a glucocorticoid and to inhibit the metabolism of catecholamines with a monoamine oxidase inhibitor also failed to demonstrate  $^3\text{H}$  adrenaline synthesis.  $^3\text{H}$  noradrenaline was found in all experiments when  $^3\text{H}$  DOPA had been given. The amounts were about 10 times higher after injection into the anterior chamber than after injection into the vitreous.  $^3\text{H}$ -dopamine was found in all except a few experiments after  $^3\text{H}$  DOPA injection. The highest amounts were found after injection into the vitreous. In experiments with  $^3\text{H}$  tyrosine we were unable to demonstrate any catecholamine synthesis. This prompts the question of whether tyrosine hydroxylase may be absent in the eyes of certain animal species.

Adrenaline in 1-2% solution is widely used as a pressure lowering drug in chronic simple glaucoma (Becker and Shaffer 1965). The action seems to be complex (Bill 1969, Kupfer et al 1971). Therefore it seemed of interest to investigate whether possibly adrenaline is formed locally in the eye as a local hormone or as a transmitter. The present report deals with attempts to find adrenaline after local injection of precursors. None was found but other interesting findings were made.

### Materials and Methods

Three catecholamine precursors were used. L-tyrosine (side-chain 2,3- $^3\text{H}$ ) was obtained from the Radiochemical Centre, Amersham, England. Its specific activity was 15.6 Ci/mmol. L-tyrosine-3,5- $^3\text{H}$  with a specific activity of 48 Ci/mmol and L-3,4-dihydroxyphenylalanine ( $^3\text{H}(\text{G})$ ) with a specific activity of 7.3 Ci/mmol were obtained from New England Nuclear Corp., Frankfurt/Main, Germany. The radio-purity of each new batch was checked by paper chromatography using n-butanol, acetic acid and water (5:1).

The purity was always greater than 95% and the amount of DOPA in the tyrosine batches was always less than 1%. Preexperimentally 250  $\mu$ Ci of  $^3$ H tyrosine or  $^3$ H DOPA were given i.v. to 4 rabbits in order to check that  $^3$ H adrenaline could be synthesized from the precursors used in the experiments. The rabbits were killed 1 h later and the amounts of  $^3$ H adrenaline in the adrenals were analysed.  $^3$ H adrenaline was found in all experiments. The  $^3$ H activities bound to adrenaline were 1180 cpm and 1130 cpm per pair adrenals in the tyrosine treated animals, 1840 cpm and 1940 cpm in the DOPA treated animals. These values are in good agreement with those reported by Kucifiansky *et al.* 1971 in the rat.

For pretreatment were used fluoxyprednisolone 16,17 acetamide 40 mg/ml (Kenacort T<sup>®</sup> Squibb) and nialamide (Niamid<sup>®</sup>) Pfizer.

The experimental animals were albino rabbits with an average weight of about 2 kg and three vervet monkeys (*Cercopithecus aethiops*). Two of the monkeys used for steroid experiments had a weight of about 1 kg and the third 4.9 kg. The animals were divided in 6 different experimental groups.

I. Fifteen previously untreated rabbits were given L tyrosine (side-chain  $^3$ H) 10  $\mu$ Ci eye. The substance was injected with a fine syringe into the anterior chamber of one eye (9 animals) or into the vitreous (6 animals). The injected volume was 10  $\mu$ l eye. The other eye served as an untreated control eye. The rabbits were killed with an overdose of Nembutal<sup>®</sup> and the eyes were immediately enucleated. The experimental time before killing the animals was from 1/2 h up to 7 days. The injected eyes and the control eyes were pooled and analysed for each analysis. One previously untreated monkey received 10  $\mu$ Ci eye of the same substance into the anterior chamber of one eye and into the vitreous of the other. The monkey was killed after 4 h and each eye was analysed separately.

II. Three previously untreated rabbits were given L tyrosine  $^3$ H 10  $\mu$ Ci into the anterior chamber of both eyes. The injected volume was 10  $\mu$ l eye. The animals were killed after 1 and 4 h. One pair of eyes was used for each analysis.

III. Eight previously untreated rabbits received L 3,4-dihydroxyphenylalanine ( $^3$ H(G)) 5  $\mu$ Ci eye into the anterior chamber of one of the eyes. This was given in a volume of 5  $\mu$ l. The other eye served as an untreated control. Half of the animals were killed and enucleated after 1 h and half of them after 4 h. Two injected eyes were pooled for each analysis so that the total precursor amounted to 10  $\mu$ Ci per sample. The control eyes were also pooled and analysed.

IV. Seven rabbits were pretreated with Kenacort T III mg/kg b.w.t. in a single dose i.m. 5 days before injection of 10  $\mu$ Ci  $^3$ H DOPA in each eye. 5 of these rabbits got the injection into the anterior chamber and 2 of them into the vitreous. 4 rabbits were given Kenacort T and  $^3$ H DOPA in the same doses and at the same time as above but 1 h before injection of the  $^3$ H DOPA into the anterior chamber of the vitreous they received 100 mg/kg b.w.t. of nialamide i.p. 4 rabbits got as the only pretreatment nialamide 100 mg/kg i.p. 1 h before injection of  $^3$ H DOPA into the anterior chamber or the vitreous. The animals in this group were killed 1 and 4 h after the injection of catecholamine precursor. One pair of eyes from each animal formed one sample in the further analyses.

V. Two monkeys were pretreated with Kenacort T 10 mg/kg b.w.t. i.m. in a single dose 5 days and nialamide 100 mg/kg i.p. 1 h before the injection of the eye. One of these monkeys received 5  $\mu$ Ci  $^3$ H DOPA and the other 5  $\mu$ Ci L tyrosine  $^3$ H into the anterior chamber of each eye. The animals were killed with Nembutal<sup>®</sup> after 4 h. Each eye was analysed separately.

#### Anal. method

For the determination of  $^3$ H adrenaline ( $^3$ H A),  $^3$ H noradrenaline ( $^3$ H NA) and  $^3$ H-dopamine ( $^3$ H DA) in the eyes we used in a slightly modified version a double isotopic derivatization technique described by Rentzhog (1971). The original technique was developed for estimation of endogenous nonradioactive catecholamines and the substance is introduced in the catecholamine molecules by acetylation with  $^3$ H acetic anhydride. In order to determine only the  $^3$ H labeled catecholamines that had been synthesized from  $^3$ H precursors we introduced the acetylation with non-labeled acetic anhydride. The different steps of the analytical procedure are given below (see Rentzhog 1971 for detailed description).

A. The enucleated eyes were trimmed and sectioned so that an "eye sample" consisting of the retina, the iris and the vitreous from 1 eye was obtained. The eye samples were homogenized in 0.4% perchloric acid containing 1% EDTA in an acid buffer. Before homogenization 10,000 cpm of adrenaline  $^3$ H was added to each sample as internal standard.

B. Alumina column separation with a batch procedure was used for primary isolation of the catecholamines.

C. After elution with formic acid and freeze-drying of the eluate the catecholamines were acetylated in an aqueous solution with non-labeled acetic anhydride to give more stable derivatives.

TABLE I An example of the  $^3\text{H}/^{14}\text{C}$  ratios after the different chromatographic steps and of the calculation of cpm  $^3\text{H}$  initially bound to catecholamines synthesized from the  $^3\text{H}$  precursors. No  $^3\text{H}$  was left in the adrenaline triacetate sample after the second chromatographic run (P4) indicating that no  $^3\text{H}$  adrenaline had been synthesized. The  $^3\text{H}$  activities present as  $^3\text{H}$  noradrenaline and  $^3\text{H}$  dopaminetriacetate after the first chromatographic run (S1) could be calculated from the cpm  $^3\text{H}$  in the final noradrenaline and dopaminetriacetate spots respectively and from the recovery of  $^{14}\text{C}$  noradrenaline and  $^{14}\text{C}$ -dopaminetriacetate in these spots. The cpm  $^3\text{H}$  initially bound to noradrenaline and dopamine were then calculated from the recovery of  $^{14}\text{C}$  adrenaline after the first chromatographic run.

Sample	Added $^{14}\text{C}$ -A cpm	$^3\text{H}/^{14}\text{C}$ ratio after chrom S1	recovery $^{14}\text{C}$ A after S1	Added $^{14}\text{C}$ NA triac or $^{14}\text{C}$ DA triac cpm	$^3\text{H}/^{14}\text{C}$ ratio after chromatography	recovery $^{14}\text{C}$ NA triac or $^{14}\text{C}$ DA triac	Final $^3\text{H}$ cpm	Calculated $^3\text{H}$ cpm after S1	Calculated $^3\text{H}$ cpm initially
					P4	S-S3 S5 S7			
IV I	10 000	A 11.75	65		-0.06	-0.03		0	0
	NA			4 800	31.0	10.5	10.1	10 900	47 400
	DA			7 700	1.15	1.14	10.4	6 050	73 000
									13 500

D Non labelled adrenaline, noradrenaline and dopamine triacetates were added as carriers.

E The triacetyl derivatives were extracted into chloroform and separated by silica gel TLC in system S1. An aliquot of the eluate from the adrenaline triacetate spot was counted by liquid scintillation. The recovery of  $^{14}\text{C}$  A after this chromatography passage was taken as a rough estimate of the losses also of NA and DA up to this analytical step.

F As a standard in the following purification step a known amount of  $^{14}\text{C}$  NA triacetate was added to the eluate from the NA triacetate spot and  $^{14}\text{C}$  DA triacetate to the eluate from the DA triacetate spot. The A, NA and DA triacetates were then rechromatographed separately in different solvent systems and small aliquots of the eluate were counted by liquid scintillation after each chromatographic run. The chromatographic purification was continued until constant  $^3\text{H}/^{14}\text{C}$  ratios were obtained indicating that pure substances had been isolated. Second acetylation to tetraacetates of the NA triacetate and A triacetate was performed for further confirmation that pure catecholamines had been isolated.

Chromatography systems listed in the order in which they were applied.

S1 Chloroform: acetic acid (1:1) Silica gel TLC

P4 Toluene: ethylacetate: methanol: water (10:1:5:5) (organic phase)

Paper chromatography (Whatman No. 1)

S3 Chloroform: methanol (9:1) chloroform: acetic acid (1:1)

Two dimension silica gel TLC

S5 Chloroform: cyclohexane: acetic acid: methanol (3:3:1:1) Silica gel TLC. This system was only used in those cases where the  $^3\text{H}/^{14}\text{C}$  ratios were not constant between P4 and S2-S3.

S8 Chloroform: acetic acid (4:1) Silica gel TLC. This system was used for the tetraacetylated catecholamines.

G Calculation. The amounts of H A in the eyes were calculated from the cpm  $^3\text{H}$  and the recovery of  $^{14}\text{C}$  A standard after the last chromatography. The amounts of  $^3\text{H}$  NA and  $^3\text{H}$  DA in the tissues were calculated from the cpm  $^3\text{H}$  and the recovery of  $^{14}\text{C}$  NA triacetate/ $^{14}\text{C}$  DA triacetate after the final chromatography and from the recovery of  $^{14}\text{C}$  A after the first chromatographic run. An example of the calculation is shown in Table I.

## Results

In experimental group I where previously untreated rabbits and one monkey were given about  $6.6 \cdot 10^{-6}$  mol of L-tyrosine (side-chain 2,3- $^3\text{H}$ ) into the anterior chamber or vitreous we could not detect any synthesis of A, NA or DA from the radioactive p- $^3\text{H}$ .

TABLE II  $^3\text{H}$  activities calculated to be present as  $^3\text{H}$  noradrenaline in one eye after local injection of  $^3\text{H}$  DOPA. Each value corresponds to one analysis (Two pooled or one separately analysed eye). The values are given as percentage of the injected  $^3\text{H}$  DOPA activity and as  $\text{mol} \times 10^{-12}$  NA. Different amounts of  $^3\text{H}$  DOPA were given in the 3 experimental groups

Exp group	Species	Pretreatment	Inj. into the anterior chamber (exp. time h)			Inj. into the vitreous (exp. time h)		
			1	4	24	1	4	
			$10^{-12}$ mol	$10^{-12}$ mol	$10^{-12}$ mol	$10^{-12}$ mol	$10^{-12}$ mol	$10^{-12}$ mol
III	Rabbit	None	117.87 037.26	088.63 040.28				
IV	Rabbit	Kenacort T	067.94 074.104	079.110 140.196	011.15	009.13	006.08	
IV	Rabbit	Kenacort T + nisalimide	043.60	014.20		003.04	001.01	
IV	Rabbit	Nisalimide	0.8.39	021.29		003.04	00.03	
V	Monkey	Kenacort T nisalimide		101.35 109.38				

Similar findings were made in experimental group II where previously untreated rabbits got about  $2.2 \cdot 10^{-10}$  mol of L-tyrosine  $3.5 \cdot 10^5$   $^3\text{H}$  into the anterior chamber. The lowest over all recoveries in the analysis were 10% for NA and A and 30% for DA. This means that the lowest detectable amounts of  $^3\text{H}$  A and  $^3\text{H}$  NA were  $6.6 \cdot 10^{-14}$  mol in group I and  $2.1 \cdot 10^{-13}$  mol in group II. The values for  $^3\text{H}$  DA were  $2.2 \cdot 10^{-14}$  mol and  $0.7 \cdot 10^{-14}$  mol respectively. This corresponds for A and NA to 0.01% and for DA to 0.003% of given amount of precursor.

In group III previously untreated rabbits were given about  $7 \cdot 10^{-10}$  mol of  $^3\text{H}$  DOPA into the anterior chamber of one eye and no treatment in the other eye. No  $^3\text{H}$  A or  $^3\text{H}$  DA was found in these samples but synthesis of  $^3\text{H}$  NA was seen. The amounts of  $^3\text{H}$  activity bound to NA in the tissues were calculated to 117% and 037% of the injected  $^3\text{H}$  activity in the two rabbits killed 1 h after the injection. The figures for the two animals killed 4 h after the injection were 089% and 040% (see top line Table II). These figures correspond to  $8.2 \cdot 10^{-12}$  mol and  $2.6 \cdot 10^{-12}$  mol (1 h) and  $6.3 \cdot 10^{-12}$  mol and  $2.8 \cdot 10^{-12}$  mol of NA (4 h) from the injected amount  $7 \cdot 10^{-10}$  mol of DOPA. Small amounts of tritium were lost from the generally labelled  $^3\text{H}$  DOPA during the synthesis and these figures therefore are slightly underestimated. No  $^3\text{H}$  NA was found in the control eyes. The lowest detectable amount of  $^3\text{H}$  A and  $^3\text{H}$  NA in this group was  $14 \cdot 10^{-14}$  mol and of  $^3\text{H}$  DA  $4 \cdot 10^{-14}$  mol.

The rabbits in group IV were pretreated with glucocorticoid glucocorticoid MAO inhibitor or only MAO inhibitor before injection of about  $14 \cdot 10^{-10}$  mol of  $^3\text{H}$  DOPA into the anterior chamber or the vitreous. No  $^3\text{H}$  A was found in these samples. All samples contained both  $^3\text{H}$  NA and all except one  $^3\text{H}$  DA. Table II shows the  $^3\text{H}$  activity calculated to be present as  $^3\text{H}$  NA and Table III the  $^3\text{H}$  DA values. The highest amounts of  $^3\text{H}$  NA were found 1 and 4 h after DOPA injection into the anterior chamber and after pretreat-

Table III  $^3\text{H}$  activities calculated to be present as  $^3\text{H}$ -dopamine in one eye after local injection of DOPA. See text Table II

Exp group	Species	Pretreatment	Inj into the anterior chamber (exp time h)			Inj into the vitreous (exp time h)		
			1	4	24	1	4	24
			$10^{-12}$ mol	$10^{-12}$ mol	$10^{-12}$ mol	$10^{-12}$ mol	$10^{-12}$ mol	$10^{-12}$ mol
III	Rabbit	None	<0.003			<0.003		
IV	Rabbit	Kenacort T	0.11 1.5 0.13 1.8	0.04 0.6 0.06 0.8	<0.003	0.83 11.6	0.25 3	
IV	Rabbit	Kenacort T + nialamide	0.11 1.5	0.17 2.4		0.90 1.6	1.30 18.2	
IV	Rabbit	Nialamide	0.20 2.8	0.14 2.0		0.47 6.6	1.31 18.5	
V	Monkey	Kenacort T + nialamide		0.007 0.04 0.004 0.014				

ment with Kenacort T. These values however were about equal to those found for  $^3\text{H}$  NA in the untreated group III. Injection of  $^3\text{H}$  DOPA into the vitreous gave about 10 times lower  $^3\text{H}$  NA values than if precursor was given into the anterior chamber.

In contrast to the findings with NA the highest values of  $^3\text{H}$  DA were found after injection into the vitreous. Lower values were noted after injection into the anterior chamber. There was no difference in  $^3\text{H}$  DA values between the 3 different pretreatments (see Table III).

Group V consisted of 2 monkeys pretreated with glucocorticoid and MAO inhibitor. One of them was given about  $5.5 \times 10^{-10}$  mol of tyrosine  $^3\text{H}$  and the other one about  $35 \times 10^{-10}$  mol of  $^3\text{H}$  DOPA into the anterior chamber of each eye. Similarly to the rabbit findings we found no synthesis of any  $^3\text{H}$ -catecholamine after tyrosine  $^3\text{H}$  DOPA gave no  $^3\text{H}$  A but both  $^3\text{H}$  NA and  $^3\text{H}$  DA were found (see Table II and III bottom line). The  $^3\text{H}$  NA values agreed reasonably well with those in rabbits but there was much less  $^3\text{H}$  DA.

### Discussion

In a previous report (Rentzhog 1972) the difficulties of identifying and measuring small quantities of one catecholamine in the presence of high concentrations of others in a tissue were discussed. The same problem arises in studies of catecholamine synthesis from radioactive precursors where several metabolites—some of them perhaps unknown—and high concentrations of other catecholamines may obscure the synthesis of a number of catechols in low concentrations. The radioactive substances formed in precursor experiments are usually separated by column and/or thin layer chromatography. Roberts (1966) and Forrest *et al.* (1970) have shown that chromatography of catecholamines may also display false double spots with Rf values close to those of other catecholamines. It is desirable to convert the catecholamines to more stable derivatives so that a careful separation will



veral different purification procedures may take place to allow detection of catecholamines which have been synthesized in very small quantities. Rentzhog (1972) has described a double isotope method for assaying endogenous catecholamines. With this technique the catecholamines are acetylated so as to obtain more stable and easily separated derivatives. In the original method this was done with radioactively labelled acetic anhydride. In the present study cold acetic anhydride was used instead and the double isotope ratio necessary for following the purification procedure was obtained from the ratio between the  $^3\text{H}$  activity of the substances formed from the  $^3\text{H}$  precursors and the  $^{14}\text{C}$  activity from added standard catecholamines. A constant  $^3\text{H}/^{14}\text{C}$  ratio in successive chromatographies in different systems indicates that a pure catecholamine has been isolated. The finding that the  $^3\text{H}/^{14}\text{C}$  ratio was unaltered after reacylation (see Rentzhog 1972) was regarded as further evidence of purity. From the recovery of  $^{14}\text{C}$  standards the losses of  $^3\text{H}$  catecholamines during the purification procedure could be calculated and compensated for. An example of the  $^3\text{H}/^{14}\text{C}$  ratio after the different steps of purification and of the calculation of recoveries are presented in Table I.

The possibility that adrenaline may be synthesized outside the adrenal glands and perhaps serve as a transmitter has been discussed (Rentzhog 1972). Several investigators have demonstrated adrenaline in rat brain by biochemical methods (Vogt 1954, Gunne 1967, Rentzhog 1972). Experiments by Ciaranello *et al.* (1969), Pohorekey *et al.* (1969) and Koslow and Schlumpf (1974) also appeared to show that enzymatic synthesis of adrenaline took place in the brain of several animal species. Hokfelt *et al.* (1973) found that certain neurons in the reticular formation of the rat contained an enzyme which produced antibodies as an immunological response to adrenal phenylethanolamine N-methyltransferase (PNMT). Saavedra *et al.* (1974) have given further evidence for the localisation of PNMT in several rat brain nuclei. L'Hermite (1971) gave systemic injections of  $^3\text{H}$  tyrosine and  $^3\text{H}$  DOPA to rats and found adrenaline in the iris and ductus deferens. Unfortunately he did not test adrenalectomized animals and the possibility that the adrenaline found in the eyes was taken up from the blood stream cannot be excluded. In our experiments we therefore considered it important to give the precursors in local injections. Neither in the rabbit nor in the monkey however did we find any evidence of adrenaline synthesis in the eye.

Wurtman and Axelrod (1965) have shown that the PNMT activity in the adrenals is controlled by cortisol and corticosterone secreted by the adrenal cortex. It might be possible therefore that circulating corticosteroids may also influence adrenaline synthesis if such takes place in peripheral neurons. We were unsuccessful however in demonstrating any adrenaline synthesis in the eye following pretreatment with a high dose of steroid. An attempt with inhibition of the metabolism of the newly formed catecholamines with a monoamine oxidase inhibitor also failed to demonstrate adrenaline synthesis.

When  $^3\text{H}$  DOPA was injected as a precursor both into the anterior chamber of the eye and into the vitreous, synthesis of  $^3\text{H}$  noradrenaline was obtained as expected. About 10 times higher quantities of  $^3\text{H}$  NA were measured after injection into the anterior chamber. The reverse was found for the synthesis of  $^3\text{H}$ -dopamine. In fact, in 2 expts. on untreated rabbits no  $^3\text{H}$  DA was detected 1 and 4 h after injection into the anterior chamber. Following pretreatment both with glucocorticoid and with nialamide however  $^3\text{H}$  DA synthesis

was established even on injection into the anterior chamber. The greater formation of DA following injection into the vitreous and of NA following injection into the anterior chamber is well in accord with the distribution of catecholaminergic neurons. The retina seems to be rich in dopaminergic cells (Haggendal 1965) while the iris is rich in noradrenergic nerve endings.

A surprising result of our studies was that when  $^3\text{H}$  tyrosine was given as precursor no synthesis of either  $^3\text{H}$  DA or  $^3\text{H}$  NA was established in either animal species. The possibility that our  $^3\text{H}$  tyrosine was inactivated was excluded by the finding in rabbit experiments that  $^3\text{H}$  tyrosine given i.v. resulted in formation of  $^3\text{H}$  adrenaline in the adrenals. This prompts the question of whether tyrosine hydroxylase (TH) may be absent in the eyes of certain animal species. In a series of preliminary experiments Palmberg and Podos (personal communication 1972) found that there was no detectable TH in an iris-ciliary body preparation from albino rabbits or brown Hartley guinea pigs. Pretreatment with reserpine that boosted the rabbit adrenal TH activity 15 fold resulted in no TH activity in the eye preparation.

Another possible explanation for our results is that tyrosine is involved to a greater extent in other metabolic pathways in the eye than in other tissues. This would mean that too little tyrosine would be available for the catecholamine synthesis. It is also possible that the quantities of tyrosine administered in our experiments were too low as injected tyrosine will be diluted by tyrosine present endogenously. Reddy *et al* (1961) found that the tyrosine concentration in the aqueous humour was  $1 \times 10^{-4}$  mol per litre. This means that in an eye the amount of tyrosine in the aqueous humour may be expected to be about  $2.5 \times 10^{-6}$  mol. The radioactively labelled tyrosine injected into the anterior chamber will therefore be greatly diluted by endogenous tyrosine resulting in a reduction of the specific activity. A dilution with the amounts of endogenous tyrosine mentioned above would reduce the lowest detectable dose of  $^3\text{H}$  NA in the eye to about  $2.5 \cdot 10^{-11}$  mol in the rabbit experiments and to about  $2 \times 10^{-11}$  mol in the experiments on monkeys. These figures may be compared with the quantities of  $^3\text{H}$  NA found in our studies following injection of  $^3\text{H}$  DOPA into the anterior chamber in the rabbit  $2.0\text{--}10.6 \times 10^{-11}$  mol and in the monkey  $35\text{--}38 \cdot 10^{-12}$  mol.

Our experiments have not shown with certainty that in these animals catecholamine can not be synthesized in the eye from tyrosine but on the basis of these findings it would seem of value to study further the sources of catecholamine synthesis in the eye of the monkey and other animals.

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## Renal Handling of Human $\beta_2$ -Microglobulin in the Rat The Importance of Sham Operation

By

UFFE RAVNSKOV and ANDRÁS KARÁTSOV

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### Abstract

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The plasma disappearance rates of human  $\beta_2$  microglobulin and (hydroxy methyl  $C^{14}$ )-inulin was measured in unoperated sham-operated ureter ligated and nephrectomised rats. Judging from the  $C^{14}$  activity curves in ureter ligated and nephrectomised rats a substantial glomerular filtration continued after obstruction of the urine flow. Almost 90% of the injected protein had disappeared from plasma 1.0 m 1 after injection into nephrectomised rats indicating a considerable extra renal elimination and invalidating attempts to evaluate different renal mechanisms of elimination before and after ureter ligation. Sham operation induced an increase of the plasma disappearance of both  $\beta_2$  microglobulin and inulin. The latter phenomenon may explain earlier findings of similarity between the disappearance curves of low molecular weight proteins in unoperated and ureter ligated animals and stresses the importance of using sham operated animals as controls.

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The elimination of low molecular weight (LMW) proteins is mainly renal. This has been demonstrated by measurements of the disappearance rate of Bence Jones proteins and  $\epsilon$ -chains (Wochner, Strober and Waldmann 1967; Mogielnicki, Waldmann and Strober 1971) parathyroid hormone (Martin, Melick and de Luise 1967) and  $\beta_2$ -microglobulin (Bernier and Conrad 1969) in laboratory animals. Such studies have shown that the disappearance rate of these proteins is similar in ureter ligated animals and unoperated controls, while it is considerably prolonged after nephrectomy. Most authors who have used this approach have suggested a mechanism of glomerular filtration of the protein followed by proximal tubular reabsorption and catabolism. They have explained the unaltered elimination rate of the LMW proteins after ureteral ligation by the filtration and reabsorption continuing uninterrupted after obstruction of the urinary flow. The degree of glomerular filtration after ureteral ligation was not measured in these studies, however. Neither was the influence of sham-operation investigated.

Measurements of the renal extraction rate of LMW proteins were additional evidence of renal elimination of these proteins. Such studies have shown that the extraction rate of insulin in dogs (Zaharko, Beck and Blankenbaker 1966) and humans (Chamberlain and

Stummler 1967 Rabkin *et al.* 1970) exceeded the filtration fraction and the relation between the extraction rate of  $\beta_2$ -microglobulin and inulin was found to be correlated with the extraction rate of p-amino-hippurate (Ravnskov Johansson and Göthlin 1972). These findings suggest a direct renal uptake of LMW proteins in addition to glomerular filtration and proximal tubular reabsorption. Such an uptake independent of the glomerular filtration rate and urinary flow could seemingly explain the high elimination rate of LMW protein in ureter ligated laboratory animals.

To explain the differences between the disappearance rates of LMW protein in normal and ureter ligated animals simultaneous measurements of the elimination of a substance known to be eliminated by glomerular filtration only was investigated. Since previous workers have used unoperated animals as controls the effect of sham-operation was studied also.

## Methods

### Experimental approach

Human  $\beta_2$ -microglobulin and  $C^{125}$  inulin were injected i.v. into 5 normal ("unoperated"), 6 sham-operated, 7 ureter ligated, and 5 nephrectomised rats and the plasma level was measured from 3 to 120 min after administration.

### Procedure

Experiments were performed on male Wistar rats weighing 250–400 g. They were allowed water *ad lib* and anaesthetised with sodium pentobarbital (4 mg/100 g b.wt.) injected into the abdominal cavity. As a rule light ether anaesthesia was added during the last 30–45 min of each experiment. The left jugular vein and carotid artery were ligated cranially and a polyethylene catheter (PE 60 Clay Adams New York) was placed in the caudal parts of the vessels. The kidneys and ureters were exposed transabdominally through a midline incision except in the unoperated group.

**Ureter ligation.** Ureters were ligated bilaterally at two places but not divided. Completeness of ligation was controlled by counting the  $C^{125}$  activity of the urine drawn from the bladder after the cessation of each experiment. Rats with urine activity greater than the activity of plasma drawn 60 min after the injection were discarded.

**Nephrectomy** was performed by ligating and by cutting of the renal pedicle.

**Sham-operation** included exposure of the kidneys and digital palpation of the ureters.

**Sampling.** 0.1–0.2 ml of a 1% solution of human  $\beta_2$ -microglobulin in an 0.01 M phosphate buffer pH 7.3 and 0.1–0.2 ml saline containing 10  $\mu$ Ci (hydroxy methyl- $C^{125}$ )-inulin per ml were injected via the indwelling jugular catheter. The non-nephrectomised rats were generally injected by more  $\beta_2$ -microglobulin than the nephrectomised to achieve measurable amounts in the plasma at the 120 min point. Blood samples of 0.3 ml were drawn via the catheter placed in the carotid artery 3, 6, 15, 30, 60 and 120 min after administration. After each sampling the catheter was washed with 0.2–0.3 ml saline containing 5 IU heparin per ml. Disposable needles and PVC syringes were used for the blood sampling.

### Substances

Human  $\beta_2$ -microglobulin was isolated from the urine of patients with severe uremia. The urine was concentrated against solid polyethylene glycol (mol. wt. 1000) using 2.5 inch dialysis tubing (Lambton Carbide) and the proteins were separated by an ion exchange chromatography on a 60  $\times$  0.9 cm column containing Whatman DE 52 equilibrated in an 0.01 M Tris HCl buffer pH 7.35 with a gradient of 0.01 to 0.1 M NaCl. The  $\beta_2$ -microglobulin-rich fractions were pooled, concentrated and further separated by chromatography on Sephadex G 75 sucrose gel equilibrated in an 0.01 M Tris HCl buffer pH 7.3 containing 0.1 M NaCl. The fractions containing  $\beta_2$ -microglobulin were dialysed against distilled water and lyophilised.

(Hydroxy-methyl- $C^{125}$ )-inulin, a substance known to be eliminated only by glomerular filtration (Shepard 1970) was supplied from the Radiochemical Centre, Amersham, England according to the

TABLE I Concentration of human  $\beta_2$  microglobulin and  $C^{14}$  activity (mean  $\pm$  S.E.) in plasma samples drawn at different time intervals after intravenous administration of the protein and (hydroxy methyl  $C^{14}$ )-inulin

		3 min (mg/l) (cpm)	6 min ( of value at 3 min)	15 min ( of value at 3 min)	30 min ( of value at 3 min)	60 min ( of value at 3 min)	120 min ( of value at 3 min)
Unoperated controls (n=5)	$\beta_2$ microglobulin	44.7 $\pm$ 13.8	63.3 $\pm$ 2.5	26.0 $\pm$ 2.2	10.4 $\pm$ 1.4	5.2 $\pm$ 0.8	2.3 $\pm$ 0.4
	$C^{14}$ activity	5689 $\pm$ 1031	71.2 $\pm$ 5.1	43.5 $\pm$ 2.4	32.1 $\pm$ 2.6	21.0 $\pm$ 2.2	14.7 $\pm$ 5.5
Sham operated (n=6)	$\beta_2$ microglobulin	36.8 $\pm$ 5.2	62.6 $\pm$ 3.3	19.2 $\pm$ 3.3	5.2 $\pm$ 1.3	2.3 $\pm$ 0.6	1.0 $\pm$ 0.3
	$C^{14}$ activity	5935 $\pm$ 939	65.1 $\pm$ 3.9	30.5 $\pm$ 3.0	16.0 $\pm$ 3.1	9.1 $\pm$ 1.8	4.4 $\pm$ 1.1
Ureter ligated (n=7)	$\beta_2$ microglobulin	31.7 $\pm$ 9.5	64.9 $\pm$ 4.2	27.1 $\pm$ 4.5	11.6 $\pm$ 2.2	5.4 $\pm$ 1.2	3.4 $\pm$ 0.7
	$C^{14}$ activity	5263 $\pm$ 827	63.6 $\pm$ 2.9	40.0 $\pm$ 3.5	9.3 $\pm$ 3.4	4.3 $\pm$ 3.0	17.5 $\pm$ 2.8
Nephrectomised (n=5)	$\beta_2$ microglobulin	23.9 $\pm$ 4.1	71.9 $\pm$ 3.7	44.8 $\pm$ 4.4	26.5 $\pm$ 5.1	18.0 $\pm$ 3.5	11.6 $\pm$ 3.4
	$C^{14}$ activity	11627 $\pm$ 3042	67.2 $\pm$ 3.2	49.6 $\pm$ 5.2	47.6 $\pm$ 5.2	38.8 $\pm$ 5.3	36.4 $\pm$ 6.1

manufacturer the specific activity was 178  $\mu$ Ci/mg the average molecular weight approximately 5200 and the radiochemical purity 99

#### Analyses

$\beta_2$  microglobulin was determined in duplicate by radial immunodiffusion as described previously (Ravnkov 1973).  $C^{14}$  activity in plasma and urine was counted in a Nuclear Chicago Mark 2 liquid scintillation counter. Before counting duplicate samples of 30  $\mu$ l were solubilised in Soluene<sup>®</sup> (Packard III, U.S.A.). The level of  $\beta_2$  microglobulin and the  $C^{14}$  activity were calculated in percentage of the value obtained in the plasma drawn 3 min after the injection.

## Results

The decrease of the plasma level of  $\beta_2$  microglobulin was considerably more rapid than that of  $C^{14}$  inulin in all groups (Table I and Fig. 1) including the nephrectomised rats. Thus the plasma half time after nephrectomy calculated from the slope of the disappearance curves between 60 and 120 min was 103 min for  $\beta_2$  microglobulin but 485 min for  $C^{14}$  inulin. Expressed in another way in comparison with the 3 min values 88.4% of  $\beta_2$  microglobulin but only 43.6% of  $C^{14}$  inulin had disappeared from plasma at 120 min.

The disappearance curves in ureter ligated and unoperated controls did not differ significantly. This was evident for both  $C^{14}$  inulin and  $\beta_2$  microglobulin. However there was a highly significant difference between ureter ligated and sham operated rats. Accordingly there was a great difference between the disappearance curves in unoperated controls and in sham operated animals. Also this difference was of a similar magnitude for  $\beta_2$  microglobulin and  $C^{14}$  inulin.

## Discussion

The considerable delay of the plasma disappearance rate of  $\beta_2$  microglobulin after nephrectomy stresses the importance of the kidney in the elimination of LMW protein. The

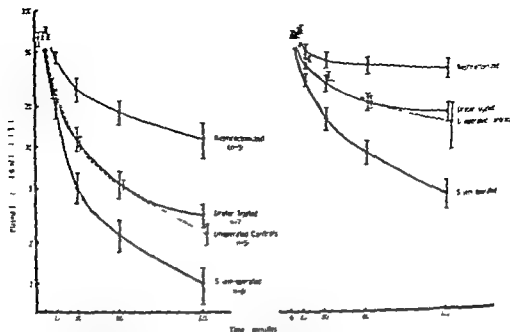


Fig. 1 Plasma disappearance curves of human  $\beta_2$ -microglobulin (left) and  $C^{14}$  inulin (right) in nephrectomized and ureter-ligated rats, and in sham-operated and unoperated controls. Bars indicate  $\pm 1$  SE. The values are given in Table I.

ference between the disappearance curves of inulin and  $\beta_2$ -microglobulin demonstrates that a substantial extra-renal mechanism of elimination also exists. Similar findings were presented by Bernier and Conrad (1969).

The elimination of  $\beta_2$ -microglobulin was less affected by ureter ligation than the elimination of inulin, apparently pointing to a direct renal uptake of  $\beta_2$ -microglobulin in addition to glomerular filtration, but the difference was small and may as well be explained in the following way. Judging from the differences between the disappearance curves of inulin in ureter ligated and nephrectomized rats glomerular filtration does not cease completely after ureteral ligation. Consequently ultrafiltrate accumulates in the tubules. Due to tubular reabsorption of water and  $\beta_2$ -microglobulin, filtration of inulin after ureteral occlusion will occur against an increasing concentration gradient, whereas no or only a minor gradient will oppose the filtration of  $\beta_2$ -microglobulin.

Earlier observations demonstrating an unchanged disappearance curve of LMW proteins after ureter ligation (Wechner, Strober and Waldmann 1967; Moggioli, Waldmann and Strober 1971; Bernier and Conrad 1969) was the stimulus to the present work. In these previous studies of the renal catabolism of proteins, ureter ligated and nephrectomized animals were compared with unoperated animals. The great difference between the disappearance rates in unoperated and sham-operated animals observed in the present study explains the high disappearance rate after ureter ligation in the mentioned works. The difference between the disappearance curves of inulin in ureter ligated and sham-operated animals in our study is also consistent with the results of Salomon and Lanza (1971) who

found that ureter ligation induced a substantial reduction but not a total disappearance of the renal extraction rate of insulin.

Half time calculations showed that the discrepancy between unoperated and sham operated rats was confined mainly to the early part of the experiments suggesting that the explanation may be an increased distribution volume in the sham-operated animals. One cause could be the formation of a traumatic edema a well known consequence of surgery (Moore *et al* 1963).

An overestimation of the elimination rate of a protein injected into abdominally operated animals may thus be introduced. Since the insulin disappearance was also increased after surgery the phenomenon is likely to be valid for any substance at least if of a similar molecular size and it stresses the importance of using sham-operated controls in such studies. Accordingly a re-evaluation of earlier data on protein elimination is needed. Previous findings of similar disappearance curves in unoperated and ureter ligated animals cannot be taken as evidence of a direct renal uptake. This mechanism has not been excluded by the present study but its existence should be demonstrated by other methods.

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# The Effect on Total Renal and Tubular Function and Plasma Renin of a Moderate Isotonic Saline Load in Rats Anesthetized with Amytal and Inactin

By

PAUL P. LEYSSAC, OLE FREDERIKSEN and SANDFORD L. SKINNER<sup>1</sup>

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## Abstract

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The renal effects of i.v. saline loading equal to 1% body weight (b.wt.) were studied in two groups of rats. Group I was anesthetized with Amytal (15 mg/100 g b.wt. plus supplementary doses). Group II with Inactin (12.5-15.0 mg/100 g b.wt.). In group I the saline load caused an increase in urine flow (+9%), solute excretion (+67%), inulin clearance ( $C_{IN}$ ) (+14%), PAH clearance (+31%) and absolute proximal reabsorption rate (+27%). Proximal fractional reabsorption and filtration fraction (FF) remained unchanged while plasma oncotic pressure (COP) decreased by 10%. Plasma renin fell and there was an inverse relationship between renin concentration and proximal reabsorption rate. In group II urine flow and solute excretion increased after saline (+85% and 110% respectively).  $C_{IN}$  and absolute proximal reabsorption rate was lower than in group I and failed to increase after saline. Proximal fractional reabsorption was also lower and decreased after saline. COP as well as renin decreased as in group I but no relationship between renin concentration and proximal reabsorption rate could be demonstrated. The data indicate that Inactin depresses both resting proximal tubular reabsorptive capacity and the tubular response to a physiological volume expansion. The results are compatible with the hypothesis that the renin-angiotensin system is significantly involved in regulation of proximal tubular function while they are in compatible with the idea that peritubular COP plays any major role in this adjustment.

It has long been recognized that the administration of isotonic saline increases the glomerular filtration rate (GFR) in the dog without change in filtration fraction (FF) (Smith 1951) and that the accompanying rise in the rate of excretion of water and solutes is smaller than the change in their rates of filtration. This observation indicated that tubular reabsorption rate of solute and water must have increased during the rise in GFR with saline administration. However, later micropuncture studies have consistently demonstrated in the dog that isotonic volume expansion to 4% of body weight (b.wt.) depresses not only fractional but also absolute reabsorption rate by the proximal tubule (Dirks *et al.* 1965; Davis *et al.* 1970).

<sup>1</sup> Visiting scientist, present address: Department of Physiology, University of Melbourne, Australia.

Mandin *et al* 1971 Herrera Acosta *et al* 1972 Wen *et al* 1973) In the rat most investigators have drawn the same conclusion in micropuncture studies with isotonic volume expansion to about 10% of b wt (Bank *et al* 1969 Brenner *et al* 1971 Daugharty *et al* 1972 Bartoly and Earley 1972 Stein *et al* 1973) More moderate isotonic saline loading has also been reported to depress absolute proximal reabsorption rate in the rat (Davidman *et al* 1972 Brenner and Berliner 1969)

These and similar observations have apparently given the impression that isotonic saline loading invariably depresses proximal reabsorption rate also in the rat The results are further taken as indirect support for and are generally interpreted according to the hypothesis of a key role of peritubular colloid osmotic pressure (COP) in regulating proximal tubular reabsorption (Earley *et al* 1966 Windhager *et al* 1969 Brenner *et al* 1969 1971)

However all these results in rats have been obtained in animals anesthetized with the thio-barbiturate Inactin In contrast to the oxy barbiturate Amytal anesthetic doses of Inactin have been shown to inhibit net fluid transport by proximal tubules *in vivo* (Elmer *et al* 1972) and isosmotic fluid transport by rabbit gall bladder *in vitro* (Christensen *et al* 1973) Furthermore Steven (1974 a) has recently reported that the proximal tubule wall is far less resistant to transmural pressure changes (is more collapsable) in animals anesthetized with Inactin as compared with Amytal anesthetized rats It is therefore of importance that Courtney *et al* (1965) studying rats anesthetized with the oxy barbiturate pentobarbital failed to demonstrate any inhibition of absolute proximal reabsorption rate even after extreme isotonic saline loadings to 10% of b wt Because the degree of volume expansion and both the dose and the particular anesthetic used could have contributed to these various findings the renal effects of more moderate isotonic saline loading (1% b wt) were investigated in two parallel series of rats the one anesthetized with Amytal and the other with Inactin The data show that in Amytal anesthetized rats moderate isotonic volume expansion significantly augments not only urine flow and solute excretion but also GFR and proximal reabsorption rate without changing FF With Inactin anesthesia GFR and proximal reabsorption rate fail to increase with the rise in urine flow and solute excretion rate Furthermore plasma COP was depressed by saline loading in both series Thus the data are incompatible with the peritubular COP as a major determinant of the proximal fluid reabsorptive capacity in normally hydrated and moderately saline loaded rats They also indicate that previous conclusions concerning the effect of saline loading on proximal fluid reabsorption (may) have been misleading due to the use of an anesthetic which itself depresses this process On the other hand the data are consistent with the hypothesis that the renin angiotensin system plays a role in regulating proximal reabsorption rate in rats anesthetized with Amytal

#### Methods

Male SPF Sprague Dawley rats weighing about 250 g were allowed free access to food and water prior to the experiment Anesthesia was introduced and maintained by intraperitoneal (i.p.) injections of barbiturate Group I was given sodium amobarbital (sodium Amytal) 15 mg/100 g b wt and anesthesia was supplemented when necessary with a daily 2 and at most 3 additional i.p. doses of 1.2 mg/100 g b wt Group II was given Inactin (Promonta Hamburg) Animals were selected excluding all rats which would not sleep sufficiently well for operation within 0 min at a maximum dose of 15 mg/100 g b wt The total dose administered ranged from 1.5 to 15 mg/100 g b wt

Rats were placed on a servo-controlled heated micropuncture table maintaining body temperature at about 37°C, and prepared for clearance, proximal occlusion time (OT) and transit time (TT) measurements as previously described (Leyssac 1964; Bojesen and Leyssac 1969). Polyethylene catheters were inserted into the left jugular vein for infusions and into the right carotid artery for blood sampling and continuous recording of systemic arterial pressure (BP) with a capacitance pressure transducer (Hansen S & W) and Servogor recorder.

A priming dose of 30 mg para-amino-hippurate (PAH) and/or 25–40 mg polyfructosan (Inutest, Lævostrøm) in saline was given *i.v.* and followed by a continuous infusion of 1.2 mg PAH and/or 0.3 mg polyfructosan *pr. min* in a volume of 15  $\mu$ l/min throughout the experiment. Experiments started after an equilibration period of about 45 min.

After collection of the first blood sample (about 200  $\mu$ l) 2 to 4 serial urine samples were collected into calibrated polyethylene tubing within 10–30 min for clearance determinations. OT was measured immediately after the last urine collection as previously described (Leyssac 1964). 2–3 min later when urine flow and BP had reached the preceding level 75–100  $\mu$ l 5% Lissamine green in 0.9% saline at pH 7.4 were injected *i.v.* and TT measured by the modification of Steinhausen's method (1963) as described by Gørtz *et al.* (1965). Blood samples (200  $\mu$ l) from the renal vein and the carotid artery were collected immediately after the TT measurements.

After the first control clearance period 2.5 ml 0.9% saline was given *i.v.* at a rate of 1.0 ml/min and followed by a continuous infusion of 15  $\mu$ l/min of isotonic saline. 30 min later a second clearance period was started including an initial blood sample, 3–4 serial urine collections, OT and TT measurements and final blood samples from the renal vein and the carotid artery.

After the experiment the left kidney was removed, drained and weighed. Inulin in urine and plasma was measured by the diphenylamine method of Bojesen (1952) modified for microanalysis. Osmolarity of plasma and urine was measured with the cryoscopic method of Ramsay and Brown (1955). PAH in urine and plasma was measured by the method of Smith *et al.* (1945).

Intratubular pressures were measured by the Landis method as described by Gottschalk and Mylle (1956). The pressure in the micropipette and a Hansen capacitance pressure transducer was adjusted by means of a mercury levelling bulb. All connections were stiff water-filled tubings. Proximal as well as distal intratubular pressures were measured in several convolutions during the urine collection periods. Colloid osmotic pressure (COP) of plasma was measured directly in 25–50  $\mu$ l plasma aliquots using an electronic osmometer (type HQ-66 S & E) as described by Hansen (1961).

Plasma renin was measured in 5  $\mu$ l aliquots by the method of Poulsen and Jørgensen (1974) which is based on angiotensin I trapping by antibody. The results are expressed as renin concentration defined as the enzymatic activity of renin uninfluenced by the individual variations in renin-substrate concentration.

Inulin and PAH clearance was calculated from the urine/plasma concentration ratios ( $U/P_{IN}$ ) and the volume of urine collected per minute ( $V_L$ ), divided by the kidney weight (KW) according to the conventional expression

$$C_{IN} = (U/P_{IN}) (V_L/KW) \text{ (ml} \times \text{min}^{-1} \times \text{g KW}^{-1})$$

End proximal tubular fluid/plasma inulin ratio ( $F/P_{IN}$ ) as calculated from the measured OT and proximal transit time ( $TT_{prox}$ ) according to the equation (Bojesen and Leyssac 1969)

$$\ln F/P_{IN} = TT_{prox} \cdot OT$$

Measured OT values were all corrected for 12% reflux from pars recta. Absolute rate of proximal reabsorption was calculated from the  $C_{IN}$  and  $F/P_{IN}$ . The rate of reabsorption in nephron segments distal to the proximal convolutions (lower nephron segments) was calculated as the difference between overall tubular reabsorption rate and reabsorption rate by the proximal convolutions.

Renal plasma flow (RPF) was calculated from the PAH-clearance ( $C_{PAH}$ ) and PAH extraction ( $F_{PAH}$ ) as

$$RPF = \frac{C_{PAH}}{F_{PAH}}$$

Filtration fraction (FF) was calculated as

$$FF = \frac{RPF}{C_{IN}}$$

A comparison of the absolute values of the various parameters measured in the control period between 2 groups was made by analysing the significance of difference between the mean values.

Within each group mean percentage changes were calculated as the means of the percentage changes in individual experiments

The significance of these mean percentage changes within a group as well as the significance of difference in mean changes of a parameter between two groups was calculated.

Significance of difference was analysed by student *t* test.

## Results

Table I and II summarize the mean values of the various parameters investigated in Amytal and Inactin groups respectively. The tables include both values for the control period and the means of changes observed in the steady state following a 2.5 ml saline load *iv*.

Control systemic arterial pressure was 12 mm Hg higher in rats anesthetized with Inactin than in those given Amytal ( $p < 0.05$ ). In neither group did pressure change after saline loading.

Although control urine flow was significantly lower in the Inactin group than in the Amytal group ( $p < 0.001$ ) the relative increase after saline was about the same (85% and 92% respectively). Also total solute excretion rate was lower with Inactin than with Amytal but the difference was not statistically significant ( $p > 0.05$ ). After saline infusion solute excretion increased significantly in both groups. The individual data are given in Fig. 1 and 2.

Inulin clearance averaged  $1.28 \text{ ml} \times \text{min}^{-1} \times \text{g KW}^{-1}$  in the control period of rats anesthetized with Amytal and increased about 25% after 2.5 ml saline (Table I). This increase was highly significant ( $p < 0.001$ ). In rats given Inactin control  $C_{IN}$  was significantly lower

TABLE I Group I. Data collected before and 30 min after an infusion of 2.5 ml isotonic saline in 250 g rats anesthetized with Amytal 15 mg/100 g b wt

	Control per od		N	Exp ch nge			p
				Abs			
BP (mm Hg)	103	$\pm 4$	24	-3	$\pm 3$	—	NS
U ine flow ( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	5.94	$\pm 0.77$	28	+5.31	$\pm 1.19$	+9.3 $\pm 14.7$	<0.001
Osm exer ( $\text{mosm} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	6.83	$\pm 0.99$	19	+3.69	$\pm 0.58$	+66.8 $\pm 16.9$	<0.001
$C_{IN}$ ( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	1.8	$\pm 0.06$	28	+0.5	$\pm 0.05$	+23.8 $\pm 5.1$	<0.001
$C_{PAH}$ ( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	3.95	$\pm 0.28$	13	+0.93	$\pm 0.36$	+30.7 $\pm 12.6$	<0.05
$E_{PAH}$ (%)	84.3	$\pm 1.4$	12	+1.81	$\pm 1.94$	—	NS
FF	0.95	$\pm 0.016$	12	-0.013	$\pm 0.000$	—	NS
F/P <sub>IN</sub> prox	53	$\pm 0.08$	8	+0.13	$\pm 0.08$	—	NS
Abs $\text{H}_2\text{O}$ reabs ( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	0.74	$\pm 0.04$	8	+0.18	$\pm 0.02$	+6.9 $\pm 4.4$	<0.001
Abs reabs lower neph segm ( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	0.535	$\pm 0.079$	27	+0.07	$\pm 0.040$	+1.7 $\pm 8.5$	NS
COP (mm Hg)	17.18	$\pm 0.65$	13	-1.53	$\pm 0.25$	-10.4 $\pm 1.4$	<0.001
Plasma ren n ( $10^4 \text{ GU ml}^{-1}$ )	3.78	$\pm 0.65$	8	-0.88	$\pm 0.34$	—	<0.05
Prox press (mm Hg)	1.80	$\pm 0.2$	14	+0.80	$\pm 0.3$	—	<0.05
Dist. press (mm Hg)	6.13	$\pm 0.5$	13	+0.6	$\pm 0.4$	—	NS

Data are given as means  $\pm$  S.E.

N = number of animals.

TABLE II Group II Data collected before and 30 min after an infusion of 2.5 ml isotonic saline in 20 g rats anesthetized with Inactin 1.5-15 mg/100 g b wt

	Control period	N	Exp change			p
			Abs			
BP (mm Hg)	115 ± 2	13	0	± 1.8		N.S.
Urine flow ( $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{g Kw}^{-1}$ )	1.97 ± 0.23	13	+1.31	± 0.79	+ 0.11 ± 0.10	<0.001
Osm. excr ( $\mu\text{osm} \cdot \text{min}^{-1} \cdot \text{g Kw}^{-1}$ )	3.87 ± 0.32	9	+3.70	± 0.61	+ 109.9 ± 25.9	<0.001
$C_{\text{IN}}$ ( $\text{ml} \cdot \text{min}^{-1} \cdot \text{g Kw}^{-1}$ )	0.92 ± 0.07	13	+0.12	± 0.07	+ 16.4 ± 8.0	N.S.
$F/P_{\text{IN-prox}}$	2.23 ± 0.08	13	-0.12	± 0.05		<0.05
Abs prox reabs ( $\text{ml} \cdot \text{min}^{-1} \cdot \text{g Kw}^{-1}$ )	0.50 ± 0.04	13	+0.03	± 0.05	+ 8.1 ± 8.7	N.S.
Abs reabs lower neph. segm ( $\text{ml} \cdot \text{min}^{-1} \cdot \text{g Kw}^{-1}$ )	0.413 ± 0.033	13	+0.085	± 0.036	+ 0.7 ± 8.1	<0.05
COP (mm Hg)	16.12 ± 0.29	9	-1.70	± 0.32	- 10.5 ± 1.9	<0.001
Plasma renin ( $10 \text{ GU} \cdot \text{ml}^{-1}$ )	5.13 ± 0.75	12	-1.07	± 0.69		N.S.

Data are given as means ± S.E.

N = number of animals

(0.92  $\text{ml} \cdot \text{min}^{-1} \cdot \text{g Kw}^{-1}$ ,  $p < 0.001$ ) and in contrast to group I it did not increase significantly after saline loading (Table II). The data from the individual experiments are given in Fig. 3.

End proximal tubular fluid/plasma inulin ratio ( $F/P_{\text{IN}}$ ) as a measure of proximal fractional reabsorption was 2.53 in the Amytal group and tended to increase after saline but the change was statistically insignificant. Proximal fractional reabsorption was significantly lower in the Inactin group ( $F/P_{\text{IN}}$  2.23,  $p = 0.025$ ) and it decreased significantly but moderately after saline ( $p = 0.05$ , Table II).

With Amytal absolute proximal reabsorption rate increased in parallel with  $C_{\text{IN}}$  (about 27%) the increase being highly significant ( $p = 0.001$ ). In contrast proximal reabsorption rate in the control period with Inactin was significantly lower ( $p = 0.001$ ) and it did not change after saline (Table II). This difference in response to saline between the two groups was significant ( $p = 0.05$ ). Data from individual experiments are presented in Fig. 4. There was no correlation between the dose of Inactin and either absolute reabsorption rate or the change following saline.

Reabsorption rate in segments distal to the proximal convolution (lower nephron segments) increased after saline in both groups although in the Amytal group the increase was not statistically significant. Individual data are given in Fig. 5.

Saline infusion caused a decrease in arterial plasma COP of about 10% from a control value of about 17 mm Hg. There was no significant difference between the two groups.

Plasma renin concentration decreased moderately but significantly ( $p = 0.05$ ) after saline in rats anesthetized with Amytal (Table I). Using paired values for renin concentration and proximal reabsorption rate between control and experimental (2.5 ml saline) periods with Amytal a highly significant inverse relationship was apparent between log renin concentra-

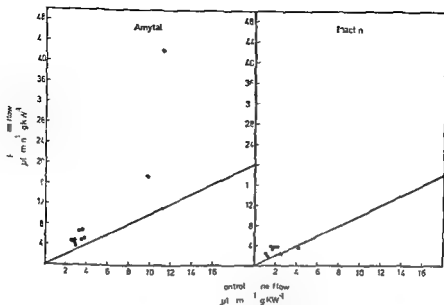


Fig. 1 Change in urine flow after 2.5 ml saline (exp. period) as compared to the control period. The line indicates line of identity. Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively.

tion and reabsorption rate (Fig. 6). In the Inactin group control renin concentrations were slightly higher, but the difference between the groups was insignificant. In contrast to the Amytal group the decrease observed after saline was not statistically significant. Nor could a relationship between renin concentration and proximal reabsorption rate be detected in the Inactin group (Fig. 6).

Intratubular pressures were measured systematically only in the Amytal group (Table I).

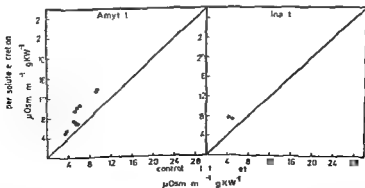


Fig. 2. Change in solute excretion rate after 5 ml saline (exp. period) as compared to the control period. The line indicates line of identity. Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively.

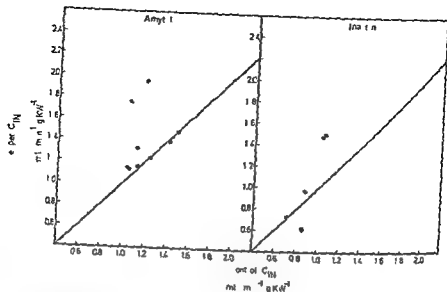


Fig 3 Change in  $C_{TN}$  after 2.5 ml saline (exp. period) as compared to the control period. The line indicates line of identity. Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively.

In the steady state following a 2.5 ml saline load both proximal and distal intratubular pressures were little altered from the control. A small but significant increase averaging 0.8 mm Hg ( $p < 0.05$ ) was observed in the proximal convolution while the mean increase of 0.6 mm Hg measured in the distal tubule was insignificant. It has however been shown that prior to the new steady state the same volume expansion causes an increase of 5.6 mm Hg in proximal intraluminal pressure (Leyssac 1969).

PAH clearance increased significantly and virtually in parallel with  $C_{TN}$  (about 10

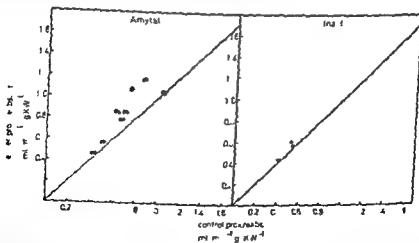


Fig. 4 Change in absolute proximal reabsorption rate after 2.5 ml saline (exp. period) as compared to the control period. The line indicates line of identity. Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively.

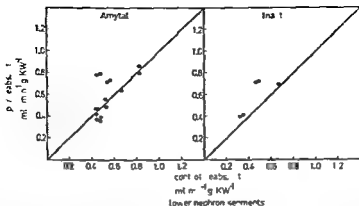


Fig. 5 Change in reabsorption rate in lower nephron segments after 2.5 saline (exp. period) as compared to the control period. The line indicates line of identity. Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively.

$p < 0.05$ ) after saline given in the Amytal group (Table I). PAH extraction ( $E_{PAH}$ ) remained unchanged and filtration fraction (FF) tended to fall; the decrease was, however, not statistically significant.

### Discussion

The present investigation deals with two topics. The first is whether or not the use of the thiobarbiturate Inactin as an anesthetic for rats has influenced the results obtained in studies on regulation of proximal tubular function. The second is whether or not the oncotic pressure of the peritubular environment is a major regulating factor for proximal tubular reabsorptive capacity under physiological conditions.

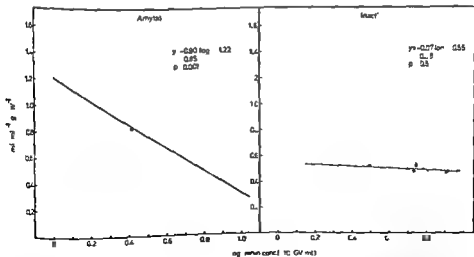


Fig. 6 Relationship between log plasma renin concentration and absolute proximal reabsorption rate in rats anesthetized with Amytal (left) and Inactin (right). Data from both control periods and after 2.5 ml saline are included. The regression lines are indicated by continuous lines. The dotted line in the diagram to the right represents the regression line obtained in the Amytal group (left).



The data demonstrate that rats anesthetized with Inactin differ with respect to renal function and respond differently to a moderate saline load than those anesthetized with Amytal.

In a previous study (Elmer *et al* 1972) using large numbers of rats it was concluded that compared with Amytal anesthetic doses of Inactin in the range 12–25 mg/100 g b wt inhibit proximal reabsorptive capacity. This conclusion was based on the following findings. Firstly a significant inverse relationship between serum Inactin concentration and proximal reabsorption rate was demonstrated. Secondly absolute as well as fractional proximal reabsorption was significantly lower in rats given Inactin and finally reabsorption rates were unrelated to serum Amytal concentration. The conclusion was supported by *in vitro* studies indicating that Inactin concentrations similar to those measured in serum of anesthetized rats caused irreversible inhibition of isosmotic net fluid transport by rabbit gall-bladder while Amytal in such concentrations did not inhibit transport (Christensen *et al* 1973). However Dev *et al* (1973) were unable to detect with micropuncture methodology any difference in single nephron filtration rate (sngfr) or proximal tubular absolute or fractional reabsorption between rats anesthetized with Inactin and Amytal nor could they find any correlation between proximal reabsorption rate and the dose of Inactin within the range sufficient for anaesthesia in their rats (10–15 mg/100 g b wt). They concluded that there was no impairment of tubular function with Inactin and further that there were no differences between the two barbiturates. Unfortunately their results are not directly comparable with our previous results (Elmer *et al* 1972) because we had to use a larger range of Inactin doses for adequate anaesthesia (12–25 mg/100 g b wt) possibly because our rats were older and larger (250 g b wt as compared to 160–220 g b wt). In addition their conclusions are based on many measurements made on few animals in each group: serum barbiturate concentrations were not measured and considerably larger supplementary doses of Amytal were given *iv* rather than *ip*.

In the present study in order to make a more valid comparison we excluded all rats which could not sleep adequately with a total Inactin dose of at most 15 mg/100 g b wt. The results show in agreement with the Munich group that within this narrow range of Inactin dose (12.5–15 mg/100 g b wt) as used by the majority of investigators proximal fractional reabsorption is slightly but significantly higher than we found previously using the larger range of Inactin doses (cf Elmer *et al* 1972) and furthermore that absolute rates of proximal reabsorption do lie predominantly within the range of values measured in rats anesthetized with Amytal (cf Fig. 4). Nor was there any detectable correlation between the dose of Inactin and the rate of reabsorption. However when the data are compared with those obtained in rats anesthetized with Amytal a depressant effect of Inactin on tubular function becomes apparent. In the Inactin group the range of proximal reabsorption rates was relatively narrow as compared to the Amytal group, all values being in the lower half of the range of reabsorption rates obtained with Amytal. Furthermore after a small saline load absolute proximal reabsorption rate (and GFR) failed to increase in rats anesthetized with Inactin in contrast to a highly significant increase in these parameters observed in rats anesthetized with Amytal. Thus even though proximal reabsorption rates similar to those in the lower range of values seen in Amytal rats can be obtained with low Inactin doses the

anesthetic blocks or severely reduces the proximal tubular response to the saline load. In contrast no inhibitory effect of Inactin could be detected in more distal nephron segments nor was the response to saline blunted in these segments. Also the diuretic and natriuretic response to saline was similar (about a doubling) in rats given oxybarbiturate and thiobarbiturate respectively. Thus the present results are in agreement with our previous observations (Elmer *et al* 1972) and are quite consistent with those reported by Dev *et al* (1973). The conclusion can be reached that the extensive use of Inactin for experimental research on regulation of proximal tubular transport is unfortunate since proximal reabsorption rate is depressed and its response to physiological stimuli is blocked by pharmacological effects of the thiobarbiturate Inactin whereas the oxybarbiturate apparently does not exert such effects.

In a previous report (Elmer *et al* 1972) an inverse relationship between log renin activity in arterial plasma and spontaneous levels of absolute proximal reabsorption rate was demonstrated in Amytal anesthetized rats. Such a relationship was absent in rats anesthetized with Inactin. In the present study induction of an increase in tubular reabsorption by moderate saline loadings raised this inverse correlation to an even more significant level than that obtained for spontaneous variations only. Such a finding is consistent with the hypothesis that the renin-angiotensin system is directly involved in regulation of proximal reabsorptive capacity and thereby of GFR, under these conditions (Leyssac 1964, 1965). Recently more direct evidence indicating an inhibitory effect of angiotensin II on proximal tubular reabsorption has been reported by Steven (1974 b) from peritubular capillary microperfusion experiments in Amytal anesthetized rats: a depression of reabsorptive capacity by angiotensin was accompanied by an increase in proximal intraluminal pressure. The opposite effect on the latter was obtained by microperfusing an angiotensin inhibitor (the competing analogue 1 Sar 8 Ala Angiotensin II) suggesting a physiological effect of endogenous angiotensin (Steven, personal communication). Finally Wiesenbaugh and Hill (1970) have shown that dogs immunized with hog renin have a diminished renal hemodynamic response to isotonic (and hypotonic) saline while excreting an increased fraction of the salt and water load as compared with normal control dogs. These data also provide evidence that the renin-angiotensin system is significantly involved in the renal response to saline loading.

Current views strongly favour an important role of the peritubular physical factors (so-called Starling factors) for the adjustment of net proximal reabsorption rate. This hypothesis would predict a depression of proximal reabsorption rate after i.v. infusion of saline because of the accompanying dilution of peritubular plasma proteins (oncotic pressure) unless a concomitant considerable rise in filtration fraction occurred. However no evidence has been provided that filtration fraction might increase following saline; on the contrary it would appear if anything to decrease. This is true for whole kidney as well as superficial nephron function (Stein *et al* 1972, Bruns *et al* 1974, Barret *et al* 1973). The present study demonstrates that in the Amytal anesthetized rat in which tubular and overall renal function is found to respond adequately without detectable drug-induced limitation, an i.v. load of 2.5 ml isotonic saline induced a 25% increment in GFR, overall tubular reabsorption and superficial proximal reabsorption rate while arterial oncotic pressure was reduced and whole kidney filtration fraction remained unchanged or fell slightly. Pressure measure-

in surface proximal tubules and adjacent peritubular capillaries have failed to yield evidence of any selective change in capillary hydrostatic pressure after isotonic volume expansion (Daugharty *et al* 1972). The present results are therefore incompatible with the concept that peritubular physical factors and in particular oncotic pressure play any significant role in adjustment of proximal tubular function after a moderate saline load. The contrary conclusion has been reached in a number of studies in Inactin anesthetized rats (see introduction). However the fact that Courtney *et al* (1965) were unable to find inhibition of proximal tubular reabsorption even after extreme volume expansion in pentobarbital anesthetized rats and also that Steven (1974 b) in similarly anesthetized rats found no change in proximal reabsorption rate during peritubular capillary microperfusion with saline (diluting the peritubular plasma) suggests that the proximal tubular response to saline and to changes in COP depends critically upon the anesthetic used.

The present results demonstrate that anesthetic doses of Inactin (12–15 mg/100 g b.wt.) depress proximal reabsorption rate in the hydropenic animal and attenuate the proximal tubular response to saline. In contrast in Amytal anesthetized rats both proximal reabsorption and glomerular filtration rates were generally higher and responded to a moderate saline load with a significant increase of about 27%. The results have implications regarding the interpretation of earlier micropuncture data obtained in Inactin anesthetized rats and indicate that Inactin should be avoided for studies on regulation of renal tubular function. In addition the data are incompatible with the physical factor hypothesis of regulation of net proximal tubular reabsorption rate but are consistent with the hypothesis that the renin-angiotensin system modulates proximal reabsorption rate under conditions of hydropenia and moderate saline diuresis in rats anesthetized with Amytal.

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## Indomethacin Blockade of Renal PGE-Synthesis Effect on Total Renal and Tubular Function and Plasma Renin Concentration in Hydropenic Rats and on Their Response to Isotonic Saline

By

P P LEYSSAC P CHRISTENSEN R HILL and S L SKINNER<sup>1</sup>

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### Abstract

LEYSSAC P P P CHRISTENSEN R HILL and S L SKINNER *Indomethacin blockade of renal PGE synthesis effect on total renal and tubular function and plasma renin concentration in hydropenic rats and on their response to isotonic saline Acta physiol scand 1975 94 484-496*

The effects of indomethacin (I), a blocker of prostaglandin (PG)-synthetase, was studied in rats in an attempt to elucidate the physiological role of renal PGE. Plasma I-concentrations of 13-14 µg/ml reduced renal venous plasma PGE-concentration significantly from 16 to 85 pg/ml within 45 min. Urine flow & solute excretion decreased by 4 and 9% respectively while urine osmolality increased 450 mOsm.  $\text{min}^{-1}$  clearance ( $C_{\text{H}_2\text{O}}$ ) and proximal reabsorption rate was unaffected while renal plasma flow (RPF) decreased by 11%. Plasma renin concentration decreased slightly but significantly. An i.v. saline load equal to 1 b.wt. given to I-treated rats failed to elevate significantly either urine flow, solute excretion,  $C_{\text{H}_2\text{O}}$ , RPF or proximal reabsorption rate but plasma renin decreased further. The normal inverse relationship between plasma renin and proximal reabsorption rate was absent. The data are consistent with the concept that intrarenal PGE plays a role in adjustment of renal vascular resistance and support the concept of a physiological role of PGE in regulating salt and water excretion. The data do not support an important physiological role of PGE in regulating proximal tubular function.

Increasing evidence suggests that renal prostaglandin  $E_2$  (PGE<sub>2</sub>) synthesized and liberated by the kidney functions as a local hormone participating in the regulation of renal blood flow and its intrarenal distribution (for review see McGiff and Irikovitz 1973). The finding of a marked reduction in overall renal blood flow following blockade of PG biosynthesis with indomethacin (Lonigro *et al.* 1973) has two important implications. Firstly, it suggests that renal vascular resistance depends upon the action of local endogenous PGE. Secondly, the marked effect of indomethacin cannot be accounted for in terms of changes in medullary

<sup>1</sup> Visiting scientist, present address: Department of Physiology, University of Melbourne, Australia

blood flow alone and therefore suggests a role for cortical PGE production. This latter suggestion is supported by the presence in renal lymph of PGE like material which increases in amounts after infusion of noradrenaline and angiotensin II (Fujimoto and Lockett 1970) and by the demonstration that not only medullary tissue but also renal cortical tissue is capable of synthesizing appreciable amounts of PGE<sub>2</sub> (Larsson and Ånggård 1973). However PGE carried to or liberated locally in the cortex will be rapidly inactivated and metabolized by its high 15 hydroxy prostaglandin dehydrogenase activity (Larsson and Ånggård 1973).

Besides its vascular and smooth muscle actions prostaglandins have been shown to influence transepithelial ion and water transport. The effect differs between high resistance and low resistance epithelia. Thus PGE enhances sodium transport but inhibits vasopressin induced water transfer across high resistance epithelia like frog skin, toad bladder and mammalian collecting duct as demonstrated first by Orloff *et al* (1965) while it inhibits net salt and water transport across low resistance epithelia such as intestinal epithelium (Alawquat and Greenough 1972; Matuchansky and Bernier 1973) and gall bladder epithelium (Leyssac *et al* 1974; Morton *et al* 1974). A moderate inhibitory effect of PGE<sub>1</sub> (but not of PGE<sub>2</sub>) on renal proximal tubular reabsorption was recently reported by Strandhoy *et al* (1974).

These findings raise the question whether or not endogenous PGE production locally in the renal cortex might be a major determinant of proximal tubular reabsorptive capacity under physiological conditions. The present investigation indicates that blockade of renal PGE biosynthesis with indomethacin in anesthetized hydropenic rats causes a reduction in urine flow and solute excretion rate without detectable effect on either glomerular filtration rate (GFR) or proximal reabsorption rate within 30 min. Furthermore indomethacin abolished the vascular, tubular and diuretic response to a moderate isotonic saline infusion without affecting the decrease in renin release.

## Methods

Male SPF Sprague Dawley rats weighing 240–290 g, allowed free access to food and water prior to the experiment, were anesthetized with intraperitoneal (i.p.) injections of sodium Amytal 15 mg/100 g b.wt. Anesthesia was supplemented when necessary with additional doses of 1–2 mg/100 g b.wt. The rats were placed on a servo controlled heated micropuncture table maintaining body temperature about 37°C and prepared for clearance, proximal tubular occlusion time (OT) and transit time (TT) measurements as described previously (Leyssac *et al* 1975).

### Group I: Hydropenic control rats

A control period including blood sampling, serial urine collections, OT and TT measurements was obtained as described in the preceding paper (Leyssac *et al* 1975). After the first control clearance period 0.75 ml of 0.9% NaCl was given, followed by a continuous infusion of 15  $\mu$ l/min. 30 min later the second clearance period was started including an initial blood sample, urine collections, OT and TT measurements and final blood samples from the renal vein and/or the carotid artery.

### Group II: Indomethacin infused hydropenic rats

The procedure was identical to that of group I except that 1.5 mg/kg b.wt. of indomethacin was included in the 0.75 ml saline, after the first control clearance period. This priming dose was followed by a continuous infusion of 15  $\mu$ g indomethacin/kg b.wt. per min in a volume of 15  $\mu$ l/min (including PAH and Polyfructosan) throughout the rest of the experiment.

### Group III 2.5 ml isotonic saline infusion to indomethacin treated hydropenic rats

Indomethacin (1.5 mg/kg b wt) was given i.v. in a volume of 0.75 ml at the time of PAH and polyfructosan priming and was followed by a continuous infusion of 15  $\mu$ g indomethacin/kg b wt per min in a volume of 15  $\mu$ l/min throughout the experiment. The procedure thereafter followed that described for group I except that after the completion of the first clearance period 2.5 ml isotonic saline were given i.v. at a rate of 1.0 ml/min followed by a continuous infusion of 15  $\mu$ l saline/min throughout the rest of the experiment.

In some of the animals of each group a 5 ml blood sample was collected from the left renal vein at the end of the experiment for prostaglandin E analysis. In order to avoid *in vivo* PGE<sub>2</sub> synthesis after the blood collection in animals not given indomethacin (group II) the blood collection cannula was connected to a 3 way stop-cock carrying one syringe containing 100  $\mu$ l indomethacin solution (0.5 mg/ml) and a second larger syringe for blood withdrawal. The indomethacin solution was gradually injected into the aspirated blood during collection assuring instant mixing and a final indomethacin concentration of about 15  $\mu$ g/ml. Blood samples were immediately cooled to 0°C and centrifuged.

After blood collections the left kidney was removed, drained and weighed.

Inulin (polyfructosan) PAH osmolality, colloid osmotic pressure (COP) and plasma renin were measured as described in the preceding paper (Leyssac *et al.* 1975).

Indomethacin in plasma was determined by a fluorimetric method based on that of Holi and Hawkins (1965). A 50  $\mu$ l aliquot of the plasma sample was diluted with an equal volume of citrate buffer pH 5.15 and thereafter extracted by shaking with 2.2 ml of n heptane containing 5% v/v pentanol for 1 min. After centrifugation 2 ml of the heptane phase were shaken with 1.1 ml of pH 11.8 Sorensen glycine buffer, centrifuged and 1 ml of the buffer phase diluted with 1 ml of the glycine buffer (total dilution 48%). The fluorescence of this solution was then measured in 1 cm-cuvettes at 29.5°C in an Aminco Bowman spectrophotofluorimeter with the excitation and emission monochromators set at 290 nm and 390 nm respectively.

Prostaglandin E<sub>2</sub> in renal venous plasma was measured either with the double isotope derivatization method of Boyesen and Buchhave (1977) or by a modification of the radioimmunoassay (RIA) method described by Caldwell *et al.* (1971). Complete details of the method will be published in a later paper but the significant modifications were as follows.

By immunization with PGE<sub>2</sub> an antibody was produced with the following cross reactivity: PGE<sub>1</sub> 100%, E<sub>1</sub> 14.1%, A<sub>1</sub> 2.8%, A<sub>2</sub> 0.7%, B<sub>2</sub> 0.3%, F<sub>2</sub> 0.06%.

**Purification of PGE.** Although the antibody was rather specific for PGE<sub>2</sub> a purification process was required before its use in RIA in order to be sure that only PGE<sub>2</sub> and E<sub>2</sub> were measured. Individual sample recovery was determined by adding <sup>3</sup>H PGE (1 000 cpm) to each 2 ml serum aliquot. The prostaglandins were then extracted from serum with acetone, concentrated by evaporation, acidified to pH 3 and re-extracted into ethyl acetate. After re-extraction with Na bicarbonate 0.1 M pH 8.3, acidification and re-extraction with ethyl acetate, chromatographic separation was performed on a micro-column of Sephadex LH 20 (10–0.5 cm) with the solvent system dichloromethane-methanol (98:2). This procedure gave a complete separation of PGE from PGA + PGB and PGF respectively.

An aliquot of the PGE fraction from each serum sample was used for recovery; the rest was used for RIA.

**RIA.** Since it was found that the eluate from the Sephadex column displaced the standard curve and that some of column-eluate without PG was added to each standard in order to correct for solvent blanks at any dose level of the standard curve. The assay procedure thereafter followed the standard procedure for prostaglandin radioimmunoassay.

Calculations and statistical analyses were performed as described in the preceding paper (Leyssac *et al.* 1975).

## Results

The dose of indomethacin which would give and maintain a plasma concentration of 10–15  $\mu$ g/ml was determined in a preliminary series of experiments. It was found that 1.5 mg of indomethacin per kg b wt followed by a continuous infusion of 15  $\mu$ g per kg b wt and 1.5 mg gave a plasma concentration of 13.6  $\pm$  2.5 (S.D.)  $\mu$ g/ml which was maintained constant for more than 2 h.

PGE<sub>2</sub>-concentration in renal venous plasma of 3 control rats (group I) measured before





TABLE II. Group II. Data collected before and 30 min after infusion of indomethacin in 250 g rats.

	Control period	N	Change after indomethacin		
			Abs	+	-
BP (mm Hg)	109 $\pm$ 2	75	-7 $\pm$ 2	—	<0.001
Urine flow ( $\mu\text{l} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	6.07 $\pm$ 0.63	40	-3.00 $\pm$ 0.53	-41.7 $\pm$ 3.4	<0.001
Osm. excret					
( $\mu\text{osm} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	8.55 $\pm$ 0.80	14	-1.73 $\pm$ 0.19	-2.03 $\pm$ 5.7	<0.001
$U_{\text{osm}}$ ( $\mu\text{osm} \times \text{kg}^{-1}$ )	1.803 $\pm$ 125	14	+450 $\pm$ 1.1	—	<0.005
$C_{\text{IN}}$ ( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	1.34 $\pm$ 0.05	16	+0.0. $\pm$ 0.07	+1.7 $\pm$ 5.1	N.S.
$C_{\text{PAH}}$ ( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	4.14 $\pm$ 0.23	7	-0.74 $\pm$ 0.14	-17.2 $\pm$ 4.8	<0.001
$E_{\text{PAH}}$ (%)	78.3 $\pm$ 3.3	7	+4.9 $\pm$ 2.7	—	N.S.
FF	0.275 $\pm$ 0.031	7	+0.054 $\pm$ 0.0.5	—	N.S.
F/P <sub>prox.</sub>	2.51 $\pm$ 0.09	40	+0.17 $\pm$ 0.11	—	N.S.
Abs. prox. reabs.					
( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	0.789 $\pm$ 0.039	16	+0.051 $\pm$ 0.050	+7.5 $\pm$ 6.5	N.S.
Abs. reabs. lower neph. segm.					
( $\text{ml} \times \text{min}^{-1} \times \text{g Kw}^{-1}$ )	0.539 $\pm$ 0.0.8	16	-0.001 $\pm$ 0.037	-0.5 $\pm$ 6.0	N.S.
Plasma renin ( $\times 10^{-4}$ GU / $\text{ml}^{-1}$ )	1.88 $\pm$ 0.25	14	-0.57 $\pm$ 0.21	—	<0.0.5

Data given as means  $\pm$  S.E.

N = number of animals

The decrease in urine flow after indomethacin was accompanied by a significant increase in urine osmolality (mean 450 mosm). Individual data for controls and treated rats are presented in Fig. 3.

Neither inulin clearance  $F/P_{\text{IN}}$ , absolute proximal reabsorption rate nor rate of reabsorption in tubular segments distal to the proximal convolution changed significantly after indomethacin. Individual data on proximal reabsorption rate obtained in the control group for comparison with those obtained in rats given indomethacin are presented in Fig. 4.

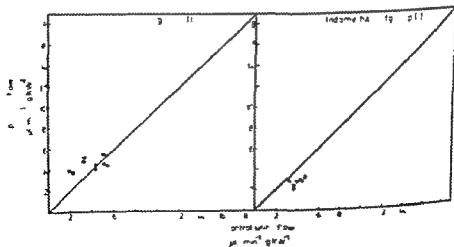


Fig. 1. Change in urine flow between period I and period II in control rats (left) and in rats given indomethacin after period I (right). The lines represent the line of identity.

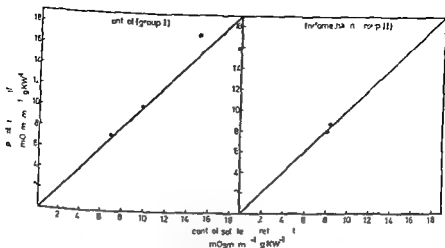


Fig. 2 Change in solute excretion rate between period I and period II in control rats (left) and in rats given indomethacin after period I (right) The line represents line of identity

Plasma renin concentration decreased after indomethacin in 13 of 14 expts. The mean change was small but statistically significant. In control rats (Table I) plasma renin tended to increase but the change was statistically insignificant. The difference in response between the two groups was highly significant ( $p < 0.005$ ).

The effect of indomethacin on renal plasma flow and filtration fraction was investigated in a small additional series of group-II experiments. RPF as estimated from  $C_{PAH}$  and  $E_{PAH}$  decreased most significantly after indomethacin.  $E_{PAH}$  did not change but  $C_{PAH}$  decreased by 17.7% ( $p < 0.001$ ) (Table II). FF was 0.275 and increased in 5 of 7 experiments to an average 0.329, a value equal to that obtained in rats treated with indomethacin from the start of the experiment (cf. Table III). However, the increase was quite variable and with the small number of experiments it was not statistically significant.

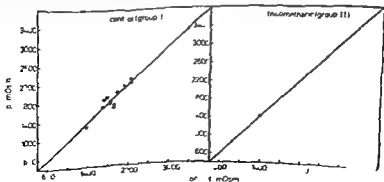


Fig. 3 Change in urine osmolality between period I and period II in control rats (left) and in rats given indomethacin after period I (right) The line indicates line of identity

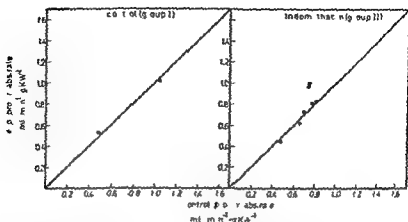


Fig. 4 Change in absolute proximal reabsorption rate between period I and period II in control rats (left) and in rats given indomethacin after period I. The line indicates line of identity.

#### Group III: Renal response to saline infusion in indomethacin treated rats

The effect of expansion of the extracellular space with 2.5 ml saline (corresponding to about 1% of b.wt.) in indomethacin treated rats (Table III) may be compared with the effect observed in a parallel series of control rats anesthetized with Amytal (Leyssac *et al.* 1975) in which the experimental procedure and conditions were identical.

Systemic arterial pressure which increased moderately during the saline infusion had returned to its control value in the experimental steady state period.

TABLE III Group III: Data collected before and 30 min after infusion of 2.5 ml isotonic saline in 50 g rats treated with indomethacin.

	Control period	N	Change after saline		
			Abs.	+	P
BP (mm Hg)	116 ±	21	-3 ± 1	—	N.S.
Urine flow ( $\mu\text{l min}^{-1} \text{ g KW}^{-1}$ )	541 ± 0.51	0	+0.9 ± 0.16	+1.156	N.S.
Solute exct. rate ( $\mu\text{osm min}^{-1} \text{ g KW}^{-1}$ )	879 ± 0.76	1	+4 ± 1.26	31.5 16.5	N.S.
$C_{\text{IN}}$ ( $\text{ml min}^{-1} \text{ g KW}^{-1}$ )	145 ± 0.06	1	+0.03 ± 0.08	5.6 5.8	N.S.
$C_{\text{PAR}}$ ( $\text{ml min}^{-1} \text{ g KW}^{-1}$ )	379 ± 0.6	8	+0.43 ± 0.00	+11.9 5.5	N.S.
$E_{\text{PAR}}$ (%)	83.6 ± 7	7	+9 ± 1	—	N.S.
FF	0.33 ± 0.017	7	+0.006 ± 0.017	—	<0.05
F/F <sub>0</sub> prox.	0.4 ± 0.09	21	0.1 ± 0.07	—	<0.05
Abs. prox. reabs. ( $\text{ml min}^{-1} \text{ g KW}^{-1}$ )	0.833 ± 0.041	1	0.08 ± 0.053	-1.63 6.9	N.S.
Abs. reabs. lower neph. segm. ( $\text{ml min}^{-1} \text{ g KW}^{-1}$ )	0.618 ± 0.015	21	-0.064 ± 0.044	-15.4 ± 7.0	N.S.
$E_{\text{osm}}$ ( $\mu\text{osm kg}^{-1}$ )	175 ± 114	1	10 ± 68	—	N.S.
COP (mm Hg)	16.9 ± 0.39	16	-1.80 ± 0.35	11.1 ± 0	<0.001
Plasma ren. a. ( $10^{-4} \text{ GU } \mu\text{ml}^{-1}$ )	90 ± 0.40	18	-0.57 ± 0.3	—	<0.05

Data given as means ± S.E.

N = number of animals.

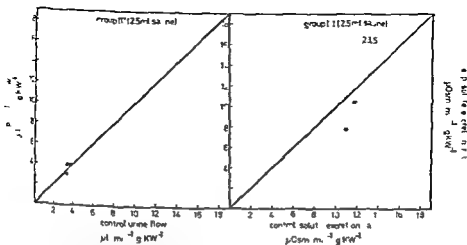


Fig. 5 Data from rats pretreated with indomethacin. Change in urine flow (left) and solute excretion (right) after 2.5 ml saline (exp. period) as compared to the control period

Urine flow and solute excretion rate increased inconsistently after saline infusion (mean +79 and +31% respectively). These increases were statistically insignificant and should be compared with the 92% and 67% increases previously reported for control rats (Leyssac *et al* 1975). The difference in urinary flow response between the present indomethacin-treated rats and previous control rats was significant ( $p < 0.01$ ) but the change in solute excretion rate was not significantly different in the present series as compared with previous controls. Individual data from the present group III are given in Fig. 5.

Inulin clearance as well as absolute proximal reabsorption rate was slightly higher in the control period of indomethacin treated rats than in both the present control group (Table I) and the previously reported control group (*cf* Leyssac *et al* 1975) but the difference was not statistically significant. However whereas  $C_{IN}$  and absolute proximal reabsorption rate increased significantly in previous control rats with 2.5 ml saline infusion both parameters failed to increase in indomethacin treated rats. The differences in response between the two groups were significant for  $C_{IN}$   $p < 0.05$  for proximal reabsorption rate  $p < 0.001$ . Individual data on proximal reabsorption rates in group III experiments are presented in Fig. 6.

Proximal fractional reabsorption as estimated from the end proximal  $F/P_{IN}$  ratio in the control period was not significantly different from that measured in control rats (period I group I). It decreased slightly but significantly following saline infusion in the present group III. Absolute reabsorption rate in lower nephron segments tended to increase after saline but the change was insignificant as it was in previous control rats.

Renal plasma flow (RPF) as estimated from  $C_{PAH}$  and  $E_{PAH}$  also failed to increase significantly in response to 2.5 ml of saline in group III rats given indomethacin.  $E_{PAH}$  was the same (84%) as in previous control rats and did not change. Filtration fraction was 0.33 a value slightly higher than but not significantly different from that in previous control rats ( $0.295 \pm 0.016$ ) and it did not change after saline infusion.

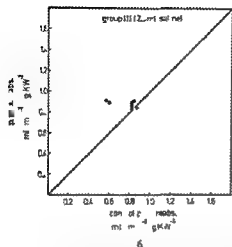


Fig. 6 Data from rats pretreated with indomethacin. Change in absolute proximal reabsorption rate after 2.5 ml saline (exp. period) as compared to the control period.

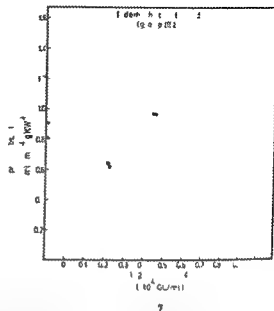


Fig. 7 Data from rats pretreated with indomethacin. Log plasma renin concentration versus absolute proximal reabsorption rate. Data both from control periods and after 2.5 ml saline are included.

Plasma renin concentration ( $2.90 \pm 0.40 \text{ IU}^4 \text{ GU/ml}$ ) was not significantly different from that measured in control rats. It decreased slightly after 2.5 ml saline, the decrease being significant at the 5% level. However, any relationship between log plasma renin concentration and proximal reabsorption rate like that previously demonstrated for control rats (Leyssac *et al.* 1975) was absent in rats treated with indomethacin, as apparent from Fig. 7. Plasma oncotic pressure averaged 16.8 mm Hg, a value not significantly different from that previously reported for control rats, also the decrease of about 10% following 2.5 ml saline was equal to that obtained in control animals.

### Discussion

In hydropenic control rats (group I) neither inulin clearance, proximal fractional or absolute reabsorption, nor urine flow and solute excretion rate changed significantly between the first and second clearance period, 30 minutes apart. If anything, the proximal reabsorption rate increased. This finding is at variance with the progressive decrease in proximal reabsorption rate after induction of anesthesia with Amytal reported by Dev *et al.* (1973). The reason for this discrepancy is probably the fact that the latter group of investigators gave higher supplementary doses of Amytal by an intravenous route during the course of their experiments than those given intraperitoneally in our laboratory.

Indomethacin, an inhibitor of prostaglandin synthetase, has been widely used as a tool to assess the physiological role of prostaglandins in various organs. However, the inter-

Interpretation of such studies is difficult because indomethacin has been shown to have effects other than PG synthetase blockade. Flores and Sharp (1972) demonstrated a 50% inhibition of cyclic nucleotide phosphodiesterase by concentrations of indomethacin similar to those obtained in the present study. Similarly Albert and Handler (1974) have shown that indomethacin (and polyphloretin phosphate) not only enhances the toad bladder response to vasopressin but also to cyclic 3'-5' adenosine monophosphate (cAMP). In the presence of exogenous PGE<sub>1</sub>, indomethacin had no effect on the response to vasopressin but still increased the response to cAMP.

In the present study indomethacin blocked PGE<sub>2</sub>-biosynthesis and release to the systemic blood. This effect was accompanied by a moderate fall in renal plasma flow (RPF) while urine flow and solute excretion rate was markedly reduced without any detectable change in the rate of glomerular filtration (GFR). This finding agrees with previous observations by Aiken and Vane (1973). Under the present antidiuretic conditions collecting duct osmolality is in near complete equilibrium with the papillary interstitium (e.g. Gottschalk and Miale 1959). The marked reduction in urine flow and solute excretion in concert with elevation of urine osmolality by only an average 450 mOsm therefore suggests increased concentrating capacity rather than urine flow reduction due solely to reduced filtration pressure. Furthermore this vasopressin-like effect of indomethacin which is directly opposite to the renal effects of exogenous PGE<sub>2</sub> (Johnston *et al* 1967; Vander 1968) is entirely consistent with the well-documented hypothesis that PGE inhibits vasopressin-induced effects on high resistance epithelia such as toad bladder and collecting duct epithelia by interference with membrane adenylyl cyclase (Orloff *et al* 1965; Grantham and Orloff 1968; Ramwell and Shaw 1970; Ozer and Sharp 1972) but the present results do not permit any distinction between the contributions to this vasopressin-like effect of potentiation of vasopressin on the collecting duct epithelium on the one hand and reduction in medullary blood flow on the other (Iskowitz *et al* 1974). Nor can it be excluded that the effect results partly from blockade of cAMP breakdown due to phosphodiesterase inhibition by indomethacin such an effect may have exaggerated the present response to blockade of PGE synthesis. However the results are consistent with the suggestion that endogenous PGE<sub>2</sub> may play a significant role in regulation of solute and water excretion in hydropenic rats.

The attenuation of the haemodynamic as well as diuretic response to a moderate isotonic expansion of the extracellular space observed in the present indomethacin-treated rats (group III) suggests that PGE<sub>2</sub> is physiologically involved in the renal tubular and/or vascular response to moderate volume expansion but again it cannot be excluded that an enhanced response to cAMP caused by indomethacin rather than a depression of PGE synthesis was responsible for this failure to respond significantly to saline. Rosenthal *et al* (1974) have shown a similarly reduced capacity to excrete salt and water in response to loading in rats deprived of PGE precursors in which pharmacological effects of indomethacin was excluded. This latter observation, therefore, lends support to the conclusion that the attenuation of the renal response to saline in rats given indomethacin is primarily a consequence of removal of endogenous PGE<sub>2</sub>.

The absence of any detectable change in absolute proximal reabsorption rate after effective blockade of PGE synthesis by indomethacin in hydropenic rats argues against a

role of endogenous PGE in the physiological adjustment of proximal tubular function under these conditions. This does not of course exclude such a role under other especially pathological conditions in which PGE release may be significantly augmented.

An unexpected finding was that indomethacin treatment of hydropenic rats resulted in a moderate but significant depression of renin release concomitant with a reduction in RPF, solute and water excretion indicating an effect of endogenous PGE and/or indomethacin on renin release from the juxtaglomerular cells. However, even in indomethacin treated rats a moderate isotonic volume expansion resulted in a further significant decrease in renin release similar in magnitude to that observed in previous control rats (Leyssac *et al.* 1975). In the indomethacin treated rats the hemodynamic (RPF) response to saline was blunted suggesting that neither PGE<sub>2</sub> nor preglomerular vasodilatation as such is required for this change in renin release.

Evidence has been previously presented supporting a role for the renin-angiotensin system in the regulation of proximal tubular reabsorptive capacity (and thereby in GFR) in non-diuretic and moderately saline diuretic rats (Leyssac *et al.* 1975). In the present study indomethacin induced a fall in renin release without detectable increase in absolute proximal reabsorption rate. Furthermore the inverse relationship between log plasma renin concentration and proximal reabsorption rate observed in control rats (Elmer *et al.* 1972) and rats given a moderate saline load (Leyssac *et al.* 1975) was absent in indomethacin treated animals. Accepting a causal relationship between the alterations in plasma renin concentration and proximal reabsorption rate in animals not treated with indomethacin, the absence of such a relationship in indomethacin treated rats indicates that indomethacin interferes with the normal proximal tubular response to local angiotensin. This might be either directly or through blockade of endogenous PGE<sub>2</sub> synthesis or other action. There does appear to be some disturbance in tubular function because whereas proximal fractional reabsorption (end proximal F/P<sub>1-4</sub> ratio) remained unchanged or tended to increase after 2.5 ml saline in Amytal anesthetized control rats (Leyssac *et al.* 1975) it decreased in indomethacin treated animals in response to an equal load (Table III). It seems worthwhile considering whether this might be due to a change in sensitivity to the local action of angiotensin, but for this to operate indomethacin would have to exert a marked sensitizing effect to the inhibitory action of angiotensin even at normal intrarenal levels of the latter. The inhibitory effect of indomethacin on nucleotide phosphodiesterase (Flores and Sharp 1972) with local accumulation of cAMP could be responsible for such an effect. Evidence favours the suggestion that elevated levels of cAMP depress fluid transfer across isosmotically transporting epithelia. Thus Fülgraff and Medforth (1974) in a micropuncture study demonstrated an inhibition of proximal reabsorption rate by cAMP and in the intestinal epithelium net transport inhibition by PGE was augmented by theophylline (Pierce *et al.* 1971) also suggesting that increased levels of cAMP inhibit isosmotic fluid transport. Evidence that transport effects of angiotensin on colonic epithelium are mediated by elevating the level of cAMP has been provided by Hornych *et al.* (1973) a conclusion apparently opposed by results reported by Davies *et al.* (1972). However, a reason for this disagreement in results could be the fact that the latter group tested effect of cAMP while Hornych *et al.* used the more permeable dibutyryl-derivative (Db-cAMP). In the rabbit gall bladder PGI<sub>2</sub> was also found to be a

potent inhibitor of osmotic transport and indomethacin potentiated the effect of added PGE about one order of magnitude (Leyssac *et al* 1974 a) This potentiation by indomethacin is again consistent with a blocking effect on phosphodiesterase

Contrary to this suggestion is our previous finding in the gall bladder that indomethacin actually depressed the inhibitory response to angiotensin (Leyssac *et al* 1974 b) However the data suggested that in the gall bladder the inhibitory effect of angiotensin was mediated by liberation of endogenous PGE<sub>2</sub> Indomethacin blocked this liberation Even though transport characteristics are very similar to those of the gall bladder the renal proximal tubule may well differ in this respect having specific receptor sites for angiotensin on the membrane bound adenylyl cyclase in contrast to gall bladder epithelium which may require other specific hormones as mediators for its transport regulation

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## Cardiovascular Responses to Changes in Carotid Sinus Transmural Pressure in Man

By

H BJURSTEDT G ROSENHAMER and G TYDÉN

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### Abstract

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To study the relative importance of cardiac and peripheral effector mechanisms in the carotid sinus baroreflex in man cardiovascular responses to equal changes of the carotid sinus transmural pressure (P<sub>tsm</sub>) in either direction of the normal were recorded and compared in eight physically well trained young male volunteers. In both the supine and the 70° head up position a decrease in P<sub>tsm</sub> produced a more potent reflex response of the systemic arterial pressure than did a similar increase in P<sub>tsm</sub>. Whereas the arterial pressure response to increased P<sub>tsm</sub> was due solely to a reduction in vascular resistance a significant increase in cardiac output contributed to the more potent pressor response to a decrease in P<sub>tsm</sub> and thus to the predominantly antihypotensive properties that characterize the carotid sinus baroreceptor control system in man. However since combined beta adrenergic and parasympathetic blockade abolished the effect of reduced P<sub>tsm</sub> on cardiac output without greatly impacting the blood pressure response it is concluded that adjustments in cardiac output are not of critical importance in the buffering function of the carotid sinus baroreceptors. Autonomic cardiac blockade exaggerated the fall in cardiac output on head up tilt, whereas arterial pressure remained unaffected due to a compensatory increase in systemic vascular resistance.

Strategically located near the base of the skull the carotid sinus baroreceptors act as an important anti-G device in man by buffering the large arterial pressure changes that would otherwise occur at head level with changes in posture. It is generally recognized that this buffer function is in part exercised by changes in total peripheral resistance mediated reflexly by variations in the tonic activity of sympathetic vasoconstrictor fibers. The extent to which reflex alterations in cardiac output may contribute to such buffering of arterial pressure changes in man is uncertain. In patients under general anesthesia Carlsten *et al* (1958) observed that electrical stimulation of the surgically exposed carotid sinus nerve produced bradycardia and decreased pulse pressure; this may have been indicative of a reduction in cardiac output. Measurements of cardiac output during similar stimulation using implanted electrodes have shown moderate decreases in this variable in unanesthetized hypertensive (Tuckman *et al* 1966) and normotensive (Epstein *et al* 1969) patients. However the effect of electrical stimulation of the sinus nerves result from both chemoreceptor and baroreceptor

tor fiber activity which complicates their interpretation. A more physiological method of stimulating the carotid sinus baroreceptors in man was first devised by Ernsting and Parry (1957) who increased the carotid sinus transmural pressure by decreasing the pressure in an airtight box enclosing the neck. These authors tested the effects of subatmospheric pressures down to  $-80$  mm Hg, but found no significant effect on cardiac output as measured on two occasions by the direct Fick method. Bevegård and Shepherd (1966) who used the same method to stimulate the carotid sinus baroreceptors found with the dye dilution technique in six subjects resting in the supine position that subatmospheric pressures in the  $-20$  to  $-60$  mm Hg range produced an average decrease in cardiac output of 10–12%.

No information is available as to the relative effects of positive and negative changes in carotid sinus transmural pressure on the cardiac output in man. Such bidirectional pressure changes can be induced by the use of a pressure/suction helmet enclosing the head as well as the neck, a method described by Thron *et al* (1967). In the present study we have used this method to assess the relative contributions of cardiac output and systemic vascular resistance to arterial pressure responses following a given change in carotid sinus transmural pressure to either side of the normal. Observations were made on healthy subjects in the supine and 70° head up position and to further study the role of cardiac function in the arterial pressure responses before and after autonomic blockade of the heart.

### Material and Method

Eight healthy physically well trained male volunteers aged 21–31 years served as subjects. Functional and dimensional data are given in Table 1.

The carotid arterial stretch receptors were exposed to different transmural pressures according to the method of Thron *et al* (1967) using a plexiglass helmet enclosing the neck and the head (Fig 1). The thoracic aperture was sealed airtight by a rubber gasket taped to the skin. The subject breathed air at normal atmospheric pressure through a mouthpiece attached to a hole in the helmet by means of a short rubber tube. To achieve the desired negative and positive pressures the helmet was connected to pressure regulators to a vacuum pump and to a tank of pressurized air respectively. A strain gauge manometer was used for monitoring of the pressure in the helmet. The time required to reach the desired pressure could be arbitrarily chosen and was kept at about 4 s.

Intraarterial pressure at heart level, cardiac output by the dye dilution method and heart rate were obtained by methods described elsewhere (Bjurstedt, Rosenhamer and Tydén 1974).

**Design of experiment.** During the course of 1–2 weeks preceding the day of the experiment at least preliminary examinations were conducted on separate days with each subject. During these sessions the subject was exposed to various positive and negative pressures over the head and neck similar to those used in the actual experiment. These periods served to accustom the subject to the subject's sensations and to the procedure.

The actual experiment commenced with a 15 min period of rest in the supine position followed by two 5 min periods during which pressures of  $+40$  mm Hg and  $-40$  mm Hg were successively applied in the helmet, separated by a 5 min period of normal atmospheric pressure. The subject was then tilted to a 70° head-up position and rested for 6 min before the above-mentioned procedure was repeated.

Six of the subjects participated in a second experimental session on a different day using the same combined para-sympathetic and beta-adrenergic blockade was induced initially by the administration of 0.03 mg/kg of atropine and 0.3 mg/kg of propranolol.

Two consecutive indicator-dilution curves were recorded in each of the supine and 70° head-up pressure periods, one after 10 and one after 1 min 30 s. Prior to these periods two control determinations of cardiac output were made in the normal atmospheric pressure in the supine as well as in the head-up position. Heart rate and arterial pressure were recorded continuously throughout the experiment.

# RESPONSE TO CHANGES IN CAROTID SINUS PRESSURE



Fig. 1 Plexiglass helmet for application of positive and negative pressures over the neck (subject breathing room air at normal atmospheric pressure through tube connected to frontal aperture)

**Calculations** Cardiac output was estimated from the mean values of the curves recorded in each experimental condition. Heart rate and arterial mean pressure were averaged during the inscription of each indicator dilution curve and in each experimental condition the mean of the averaged values was used for the calculations. Total peripheral resistance was calculated as mean arterial pressure (mm Hg) divided by cardiac output (ml/s). The statistical significance of differences between mean values were evaluated by applying the *t* test to the intra-individual differences (cf Fisher (1948)).

## Results

Table II shows the control values (means and ranges) of cardiovascular variables in the supine and 70° head up positions before and after combined parasympathetic and beta adrenergic blockade. The circulatory adjustments noted after a 40 mm Hg change in the

TABLE I Functional and dimensional data. BSA = body surface area from nomogram of Dubois and Dubois (1916); HR = heart rate after 15 min of supine rest; MAP = mean arterial pressure in mm Hg at heart level after 15 min of supine rest

	Age years	Weight kg	Height cm	BSA m <sup>2</sup>	HR		MAP	
					Supine	Head up	Supine	Head up
GT	28	73	190	1.99	64	89	100	111
IG	3	76	183	1.98	49	66	96	100
MR	-	80	190	2.07	55	9	83	89
BG	25	74	179	1.90	61	88	96	101
JP	4	63	173	1.75	71	83	99	114
MS	1	73	185	1.95	70	86	96	95
BJ	-	88	178	1.78	58	83	88	10
LL	31	67	18	1.86	59	20	93	96

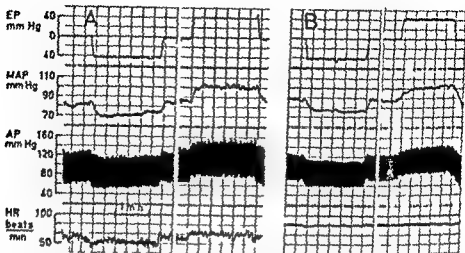


Fig. 2 Segments of recordings from subject MR showing effects of changing the external pressure over the neck (EP) on mean arterial pressure (MAP) obtained electronically from recorded arterial pressure in the radial artery (AP) and heart rate before (A) and after autonomic cardiac blockade (B).

helmet pressure positive as well as negative before and after autonomic blockade and in the supine as well as the 70° head up position are given in Table III.

#### *Responses to decreased external pressure on the neck*

**A Before autonomic blockade** A typical reflex response to a 40 mm Hg decrease of the external pressure over the neck in the supine position is shown in Fig. 2 A (left panel). Mean arterial pressure fell abruptly showing a slight overshoot and a partial recovery during the 2 min period of exposure. Heart rate also decreased and remained lowered. Average decrease in mean arterial pressure in the supine position was 8 mm Hg, corresponding to 9% decrease in heart rate. While there was no statistically significant change in cardiac output, total peripheral resistance decreased 12%.

In the 70° head up position decreased helmet pressure evoked an 8% fall in mean arterial pressure and a 9% decrease in total peripheral resistance, whereas heart rate and cardiac output showed no significant changes.

**B After autonomic blockade** Fig. 2 B (left panel) exemplifies the reflex response in the supine body position to a 40 mm Hg decrease in external pressure over the neck after combined parasympathetic and beta adrenergic blockade. Mean arterial pressure fell 8 mm Hg in the supine and 11 mm Hg in the head up position. Heart rate changes were almost abolished in both body positions. As before autonomic blockade, there were no significant changes in cardiac output. Total peripheral resistance showed a tendency to decrease, the change being statistically significant only in the head up position (Table III).

#### *Responses to increased external pressure on the neck*

Reflex responses obtained by increasing the external pressure on the neck by 40 mm Hg in the supine position before and after autonomic cardiac blockade are exemplified in Fig. 2 A and B (right panels).

TABLE II Control values of cardiovascular variables recorded in the supine and head up positions before and after autonomic blockade of the heart (neck exposed to normal atmospheric pressure)  
 MAP = mean arterial pressure in mm Hg HR = heart rate in beats/min CO = cardiac output in l/min TPR = total peripheral resistance in mm Hg sec/l

		MAP	HR	CO	TPR
Without autonomic blockade	Supine	95 (85-107)	62 (49-71)	7.6 (6.8-8.8)	1.76 (0.87)
	70° head up	102 (89-114)	84 (66-9)	5.6 (5.0-7.0)	7)
With autonomic blockade	Supine	99 (90-107)	88 (85-97)	8.8 (7.0-10)	( )
	70° head up	103 (95-117)	91 (86-100)	4.3 (3.3-4.9)	(1.1)

*A Before autonomic blockade* In general the responses of the arterial mechanism to the heart rate to 40 mm Hg increase in external pressure were stronger than they were with a corresponding decrease in external pressure. They were also accompanied by significant increases in the cardiac output. In the supine position mean arterial pressure and heart rate increased 17 and 16%, respectively while cardiac output increased 10% and total peripheral resistance 7%.

In the 70° head up position mean arterial pressure rose by 15%. Heart rate and cardiac output increased 6% and 10%, respectively while there was no statistically significant change in total peripheral resistance (Table III).

*B After autonomic blockade* Following combined parasympathetic and beta-adrenergic blockade increased external pressure on the neck caused mean arterial pressure to rise 12% in the supine and 14% in the head up position with little change in heart rate. Cardiac output was not affected either in the supine or the head-up position whereas calculated total peripheral resistance increased 14% and 15% respectively both differences being just above the  $p < 0.05$  level.

TABLE III Cardiovascular responses ( $M \pm S.E.$ ) to positive and negative changes in the external pressure on the neck (for control values see Table II)

		MAP mm Hg	HR beats/min	CO l/min	TPR mm Hg sec/ml
-40 mm Hg without autonomic blockade n=8	Supine	$-8 \pm 1$	$-40 \pm 17$	$+0.3 \pm 0.4$	$-0.09 \pm 0.04$
	70° head up	$-8 \pm 1$	$-19 \pm 11$	$0.0 \pm 0.1$	$0.10 \pm 0.04$
-40 mm Hg with autonomic blockade n=6	Supine	$8 \pm 1$	$-0.8 \pm 0.3$	$-0.1 \pm 0.1$	$-0.05 \pm 0.01$
	70° head up	$11 \pm 1$	$-1.0 \pm 0.4$	$-0.1 \pm 0.1$	$0.11 \pm 0.04$
+40 mm Hg without autonomic blockade n=8	Supine	$+16 \pm 1$	$+10.1 \pm 1.4$	$+0.8 \pm 0$	$0.05 \pm 0.0$
	70° head up	$15 \pm 1$	$+4.8 \pm 1.7$	$+0.6 \pm 0$	$0.04 \pm 0.0$
+40 mm Hg with autonomic blockade n=6	Supine	$-1 \pm 1$	$+1.3 \pm 0.4$	$-0.1 \pm 0.4$	$-0.10 \pm 0.03$
	70° head up	$-14 \pm 3$	$+0.5 \pm 0.5$	$0.0 \pm 0.3$	$+0.1 \pm 0.0$

### Discussion

When the external pressure over the head and neck is decreased the driving pressure in the enclosed vascular segments approximates the difference between inflow and outflow pressures except initially for as long as it takes for the enclosed distensible veins to fill and the pressure in these vessels to approximate the outflow pressure so that flow is resumed. Thus, when steady state conditions have been reestablished a subatmospheric helmet pressure does not affect the driving pressure in cerebral circuits. From a hemodynamic point of view this situation is however basically different from that obtaining with a positive pressure in the helmet. In the latter case the pressure in the downstream ends of the enclosed vascular segments is close to that of the surrounding pressure rather than to the outflow pressure due to the waterfall effect which characterizes the outflow from collapsible veins when the surrounding pressure is higher than the outflow pressure (cf. Permutt and Riley 1961). This means that in the present experiments with a positive pressure in the helmet the transmitted portion of this pressure was lost from the normal driving pressure (inflow pressure minus outflow pressure).

The possibility has been considered whether with a pressure of +40 mm Hg in the helmet the associated reduction of the normal driving pressure in cerebral circuits may have produced a systemic vascular response due to underperfusion and ischemia in medullary centers. The situation is hemodynamically similar to that obtaining when the cerebrospinal fluid (CSF) pressure is increased the cerebral driving pressure in this case being reduced by an amount corresponding to the surrounding CSF pressure. However, Greenfield and Tindall (1965) found that an increase in the CSF pressure in man of 68 mm Hg (with a corresponding reduction of the driving pressure in cerebral circuits) reduced cerebral blood flow by only 25% and did not affect either the arterial blood pressure or the heart rate. It is unlikely therefore that the much smaller increase in surrounding pressure produced in the present experiments by the application of a pressure of 40 mm Hg in the helmet could have provoked a cerebral ischemic pressure response. Furthermore, Sagawa, Ross and Guyton (1961) observed in dogs that with the carotid sinus nerves sectioned and the cerebral arterial circulation isolated from the systemic circulation no such response occurred until the cerebral perfusion pressure fell below 40 mm Hg.

Evidence has been presented by Thron *et al.* (1967) that with the present technique the pressure transmitted to the wall of the carotid sinuses is linearly related to the applied helmet pressure when the latter is changed in the range -60 to +60 mm Hg. By applying positive and negative pressure of 40 mm Hg in the helmet equal transmural pressure changes in opposite directions from the normal could thus be produced and their cardiovascular effects compared. The observation that decreasing the transmural sinus pressure produces a much stronger arterial pressure response than when the transmural pressure is increased by the same amount is in agreement with the results of Thron *et al.* (1967) and Stegeman, Busert and Brock (1974). The 17% rise and 9% fall in mean arterial pressure that resulted in supine subjects from changing the external pressure by 40 mm Hg in the positive and negative direction respectively are nearly identical with the results reported by these authors. In the present experiments similar results were obtained in the head up position; the cor-

responding arterial pressure changes being +15 and -8%. Our results therefore support the notion that the buffering effect of the carotid sinus baroreceptors i.e. their negative feedback role in response to a change in intrasinus pressure is clearly stronger following a fall than a rise in this pressure. That the pressure response to a given decrease in transmural sinus pressure was not stronger in head up tilt than in the supine position may be explained by the lowering of intrasinus pressure on standing up being too small to allow an increased baroreceptor responsiveness to stand out.

The observation that the carotid baroreflex acts as a predominantly antihypotensive mechanism in man refers to an intact system in which the aortic receptors tend to minimize the effects of changes in carotid baroreceptor activity. One can only speculate whether the less potent blood pressure response to an increase in transmural sinus pressure was in part due to a stronger counterregulation on the part of the aortic baroreflex. However if the finding in dogs that this reflex is relatively ineffective in buffering a decrease in arterial pressure below normal (Donald and Edis 1971, Pelletier, Clement and Shepherd 1977) also pertains to man this discrepancy in blood pressure response to changes in transmural sinus pressure to either side of the normal would reflect important characteristics of the carotid sinus mechanism in man. These characteristics differ from those obtained in anesthetized vagotomized animals with surgically exposed and isolated open loop carotid sinus preparations which have shown nearly symmetrical blood pressure responses to changes in mean intrasinus pressure on either side of the normal (for references see Heymans and Neil 1958). Intrasinus pulse pressure and  $dp/dt$  which may interact non additively with the changes in intrasinus mean pressure as determinants of baroreceptor activity (for references see Folkow and Neil 1971, Zoller *et al* 1972) may also have contributed to the observed nonlinearity of the system by exerting different effects at different levels of mean transmural pressure.

The present experiments were directed toward studying the relative importance of the cardiac and peripheral effector systems in the buffer function of the carotid sinus baroreceptors. Measurements of cardiac output showed a significant 10% increase when the transmural sinus pressure was lowered in the supine and head up position whereas no significant change occurred with a corresponding increase in transmural pressure. Thus the difference between the magnitudes of the blood pressure responses in the two cases is attributable to differences in the contributions of cardiac output and systemic vascular resistance whereas in both body positions the arterial pressure response to increased transmural sinus pressure was due solely to a reduction in vascular resistance, a significant increase in cardiac output contributed to the more potent pressor response to a decrease in transmural pressure and thus to the predominantly antihypotensive properties that characterize the carotid sinus baroreceptor control system in man. The observation that a subatmospheric pressure of 40 mm Hg over the neck did not influence cardiac output is in agreement with the results of Ernsting and Perry (1957), Bevegård and Shepherd (1966) on the other hand reported a decrease in cardiac output in six subjects with subatmospheric pressures of -20 to -60 mm Hg, but this effect was statistically significant only at -60 mm Hg. The present data indicate that reflex counter regulation of cardiac output is more easily elicited by a decrease than by an increase in transmural sinus



Combined beta adrenergic and parasympathetic blockade strongly influenced the cardiovascular adjustments following a change from the supine to the head up position. Thus, from the control values for cardiac output given in Table II it is evident that a shift from the supine to the head up position caused a much greater fall in this variable after blockade than it did before (51 and 26%, respectively,  $p < 0.05$ ). In spite of this mean arterial pressure in the head up position was not lowered and the exaggerated curtailment of cardiac output after blockade was therefore fully compensated by a greater increase in systemic vascular resistance. The question remains whether the greater fall in cardiac output following autonomic blockade was due to cardiac or peripheral effects or both. Calculated average stroke volume in the head up position was smaller after than before blockade (48 and 67 ml respectively,  $p < 0.01$ ) presumably in part due to blockade of beta adrenergic receptors which has previously been shown to diminish stroke volume in the sitting (Bjurstedt *et al.* 1974) and standing (Sannerstedt, Julius and Conway 1970) positions. Loss of baroreceptor induced sympathetic inotropic drive to the heart may have been involved (cf. Sarnoff *et al.* 1960, Glick 1971) but decreased venous return may also have contributed secondary to increased blood pooling in intracardiac capacitance vessels by the action of atropine (Kaiser, Frye and Gordon 1954, Miller *et al.* 1954) and by prevention of the venoconstriction normally mediated by stimulation of beta adrenergic receptors (cf. Kaiser, Ross and Braunwald 1963).

It can be seen in Table III that the difference in magnitude between the blood pressure responses to increased and decreased external pressure on the neck, both in the supine and head up positions, was less evident during combined beta adrenergic and parasympathetic blockade which prevented any intervention on the part of the cardiac effector mechanism. That in the absence of such intervention the reflex blood pressure responses to changes in transmural sinus pressure were not greatly impaired, however, indicates that baroreceptor induced adjustments in cardiac output are not of critical importance in the buffer function of the carotid sinus baroreceptors. This is concordant with the observation of Epstein *et al.* (1969) that electrical stimulation of the carotid sinus nerve in man causes a fall in systemic arterial pressure also when cardiac output is kept constant by mild exercise. The present finding that the baroreceptor induced blood pressure response was not greatly influenced by either posture or autonomic blockade also tallies with other results obtained with electrical stimulation of the sinus nerve in man (Eckberg *et al.* 1972).

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## Vasomotor Nerve Control of Isolated Arteries and Veins

By

BENGT LJUNG<sup>1</sup>, JOHN A BEVAN<sup>2</sup>, BARBARA L. PEGRAM<sup>2</sup>, RALPH E. PURDY and  
MARY SU

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### Abstract

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In order to compare neuro-effector function in different blood vessels, frequency response relations were determined for the following preparations: 1) Isolated rings of the proximal saphenous, distal saphenous and ear arteries, the parietal branch of the internal iliac vein and the small saphenous vein from 1 rabbit; 2) spiral strips of the rabbit pulmonary artery and 3) longitudinal preparations of the rat portal vein. In each rabbit tissue only one low (4 Hz) and one high (8 Hz) transmural nerve stimulation frequency was applied until steady state responses were obtained and these were expressed as a percentage of a maximum response to exogenous noradrenaline (NA) applied in each experiment. The general shape of the frequency response curves was similar, but differences in steepness and amplitude of the maximum neurogenic response relative to exogenous NA were found. The steepness of the frequency response relations of the veins tended to be greater than those of the arteries. It appears that factors such as the neuro-muscular contacts, presence of terminal nerve fibres within the media and the operation of mechanical factors for myogenic propagation of activity contribute to the effectiveness of neurogenic vascular control as revealed by frequency response curves. *In vivo* geometrical factors can greatly augment the hemodynamic significance of the observed differences.

The predominant extrinsic control of the vascular smooth muscle in most vascular beds is exerted via postganglionic adrenergic nerve fibres. During the last decade neurogenic vascular control has been analyzed in some detail in a select number of isolated blood vessel preparations (for ref. see Bevan and Su 1973). Large arteries and veins are primarily suited to this type of study for technical reasons.

There is considerable variation in the extent and characteristics of nervous control of the hemodynamically important small arteries and veins *in vivo*. For example Mellander (1960) found that venous capacitance responses to vasomotor nerve stimulation at low impulse rates were relatively more pronounced than arterial resistance responses in the

<sup>1</sup> Present address and address for correspondence: Department of Physiology, University of Göteborg, Fack 540033, Göteborg, Sweden.

<sup>2</sup> Present address: Department of Pharmacology, Christian-Albrechts-Universität, Hermannstrasse 48, D-23 Kiel, West Germany.

hindquarter of the cat. Variation in the nervous control of arterial resistance has also been found in different vascular circuits (for ref. see Mellander and Johansson 1968). Such quantitative inequalities *in vivo* are difficult to evaluate in terms of smooth muscle activation because of the complex interrelations and geometry of consecutive vascular sections.

In order to directly compare frequency response relationships in arterial and venous smooth muscle isolated ring preparations of different arteries and veins from the rabbit have been studied in the present experiments. Isometric force responses to electrical field stimulation at graded impulse rates were determined in the proximal saphenous distal saphenous ear and pulmonary arteries the parietal branch of the internal iliac vein and the small saphenous vein. Differences in the frequency dependence and maximum extent of the adrenergic nervous control of these vessels seem to be related to previously reported variation in their neuro-effector organization.

## Methods

A bino rabbits weighing 2.0–5 kg were stunned by a blow to the head and exsanguinated. The following blood vessels were carefully isolated and dissected under a dissection microscope and placed in a bath of oxygenated Krebs bicarbonate solution: the central aorta (proximal part) the pulmonary artery the proximal saphenous artery (at the thigh) the distal saphenous artery (above the ankle) a parietal branch of the internal iliac vein and the small saphenous vein (below the knee joint). Ring preparations were made of most vessels by cutting them in sections 3–5 mm in length. These rings were mounted under 5–10 mN passive force for recording of isometric responses according to the method described by Bevan and Osler (1972). For technical reasons spiral strip preparations were made of the pulmonary artery instead of rings.

Results from the rat portal vein previously reported in preliminary form (Ljung 1970) have been included as well for comparison. The experimental method for isolation and mounting of the longitudinal muscle preparation of the rat portal vein has been described elsewhere (see Ljung 1970).

Nerve responses were obtained by selective activation of the intramural adrenergic nerve plexus by means of transmural electrical field stimulation. A Grass S-4 stimulator provided square wave impulses of suprathreshold amplitude and 0.1-0.8 ms in duration to platinum electrodes mounted on either side of the preparation. Frequency response relationships were obtained in experiments performed according to the following protocol. The tissue was first allowed to accommodate for 10 min. Transmural field stimulation was then applied at a low frequency of either 0.5 or 4 Hz until a plateau was reached. After a resting period of 15 min a second stimulation at an impulse rate of 8 Hz or higher was similarly made to a plateau level. When the baseline had completely returned to the resting level of passive force (usually 15 min later) a maximum response to exogenous noradrenaline (NA) was induced by injection of NA (10<sup>-6</sup> M) into the bath solution. In this way a series of experiments on preparations from a certain blood vessel provided an accurate compounded frequency response curve where each preparation contributed one equilibrium response value from the low (4 Hz) and one from the high (8 Hz) impulse frequency ranges. Plateau values for some time course were expressed as a percentage of the maximum NA response and plotted against impulse frequency. In addition to the mean responses to each impulse frequency were expressed as a percentage of the maximum response to nerve stimulation in each series of experiments on a particular blood vessel.

Concentration effect curves for exogenous NA were obtained in three selected vessels (aortic strip preparations of the pulmonary artery and ring preparations of the ear artery and small sphenoid vein) by repeated injections of NA in the same concentrations into the bath for 3 min at 15 min intervals with repeated washes in between. The NA response values were expressed as a percentage of the maximum NA response (ED<sub>100</sub>) was determined graphically. The agonist concentration which gave half the maximum response (ED<sub>50</sub>) was determined graphically. In each experiment, in separate experiments the NA sensitivity was determined after blockade of the neuronal uptake by desmethylimipramine (DMI 10<sup>-6</sup> M) and after blockade of the  $\beta$  adrenergic receptors by propranolol (10<sup>-6</sup> M). The data were analysed by Student's *t* test, pairing design or by means of

Comparison of mean values was performed by Student's *t* test, pairing design or by *post hoc* analysis.

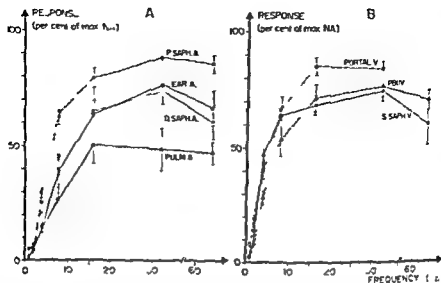


Fig. 1 Frequency response relationships obtained in isolated arteries (A) and veins (B) from the rabbit. Each preparation stimulated with one low ( $< 4$  Hz) and one high impulse frequency only. Responses expressed as a percentage of a maximum response to NA. Number of preparations: Proximal saphenous artery (P.SAPH.A.) 8, ear artery 56, distal saphenous artery (D.SAPH.A.) 78, pulmonary artery (PULM.A.) 35, parietal branch of internal iliac vein (P.BIV.) 56 and small saphenous vein (S.SAPH.V.) 44. For comparison a curve obtained in 20 rat portal vein preparations stimulated in random order (Ljung 1970) is included in Fig. 1B. Mean values  $\pm$  S.E.

## Results

Curves for the various blood vessels relating nerve stimulation frequency to the corresponding response amplitude expressed as a percentage of maximum noradrenaline (NA) response ( $10^{-4}$  M) are illustrated in Fig. 1. Their general configuration is similar in that they are sensitive in the low frequency range (1–4 Hz) of postganglionic sympathetic nerve stimulation and level off to reach maxima at 16–32 Hz. It is of interest to compare the amplitudes of the maximal neural responses and the steepness of the initial segments of these curves. The variation in the former parameter (Table 1) is apparent from Fig. 1. Of the arteries studied (Fig. 1A) the proximal saphenous artery gave the greatest neurogenic relative to the NA response and the pulmonary artery the smallest. The amplitudes for the distal saphenous and the ear arteries ranged in between these values and so did those of both the small saphenous vein and the parietal branch of the internal iliac vein (Fig. 1B). Corresponding data for the longitudinal smooth muscle of the rat portal vein (Ljung 1970) have also been included in Fig. 1B and Table 1. Its maximum neurogenic response value was as high as that of the proximal saphenous artery.

In Fig. 2 the mean responses to each impulse rate have been expressed as a percentage of the maximum neurogenic response of a particular vessel. Although the shapes of the curves for the different arteries and veins are similar their initial slopes do vary, i.e. sensitivity to changes in impulse rate within the low frequency range is different. In order to quantify these differences two findings from Fig. 2 were utilized. First, threshold stimulation frequencies were found to be essentially the same in the different vessels and

TABLE 1 Responses of isolated arteries and veins to transmural stimulation

Blood vessel	Maximum response		
	Amplitude (max. NA $\pm$ S.E.)	$f_{90}$ <sup>a</sup> (Hz)	$f_{50}$ (Hz)
Pulmonary artery	51 $\pm$ 8.1	16	14.3
Dist. saphenous artery	77 $\pm$ 3.3	37	10.3
Ear artery	74 $\pm$ 5.8	32	8.5
Prot. saphenous artery	88 $\pm$ 5.5	32	5.5
Parietal branch of internal iliac vein	77 $\pm$ 4.5	32	7.4
Small saphenous vein	75 $\pm$ 4.4	32	4.7
Rat portal vein	85 $\pm$ 3.8	16	4.9

<sup>a</sup>  $f_{90}$  and  $f_{50}$  indicate the frequencies at which maximum and half maximum neurogenic responses were obtained respectively.  $f_{90}$  was determined by linear regression.

secondly the relationship between impulse rate and response amplitude was practically linear for responses of up to 60–70 per cent of the maximum neurogenic response (Fig. 2). Therefore the value of the frequency which gave 50 per cent of the maximal neurogenic response,  $f_{50}$ , could be approximately determined by linear regression analysis and taken to represent the steepness of the frequency response curves. The obtained  $f_{50}$  values (Table 1) were lowest for the small saphenous vein and the rat portal vein and twice as high in the distal saphenous and pulmonary arteries. The frequency response curves of the veins tended to be steeper than those of the arteries. This distinction did not apply to the maximum response values. Thus it seems that the extent and characteristics of the neuro-effector control in a particular blood vessel is determined by the functional properties of its vasomotor nerve supply and smooth muscle (see Discussion).

The pulmonary and ear arteries and the small saphenous vein were selected for study of the sensitivity of the smooth muscle to exogenous NA in separate experiments. The shape and

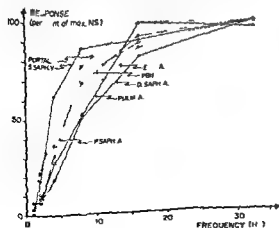


Fig. 2. Frequency response curves of different arteries and veins. Max. values from Fig. 1 expressed as a percentage maximum response to nerve stimulation.

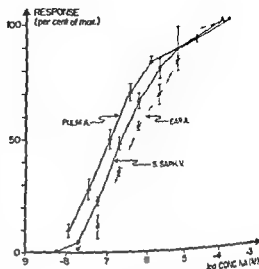


Fig. 3 Concentration-effect curves for noradrenaline (NA) obtained in experiments on strip preparations of pulmonary artery ( $n=8$ ) and in ring preparations of the ear artery ( $n=4$ ) and the small saphenous vein ( $n=4$ ). Note that in ring preparations NA will enter the tissue via the adventitial surface only.

slope of the concentration-effect curves (Fig. 3) were comparable in the three vessels. The apparent sensitivity to NA was expressed as that concentration which produced half the maximum response  $ED_{50}$  and found to be of the same order of magnitude for all three vessels in the control situation (Table II). Blockade of the neuronal uptake mechanism, by treatment with desmethylinipramine (DMI  $10^{-7}$  M) caused a 13 fold decrease in  $ED_{50}$  for the ear artery but the  $ED_{50}$  value was only reduced by a factor of 2.9 and 1.8 in the small saphenous vein and in the pulmonary artery respectively. It thus seems that the sensitivity of the effector tissue proper to excitation by NA is greater in the ear artery than in the two other vessels. Blockade of the  $\beta$  adrenergic receptors with propranolol ( $10^{-7}$  M) did not significantly alter the  $ED_{50}$  values (Table II).

### Discussion

lood vessels must react to meet varying functional demands within the consecutive sections of the circulatory system and in the parallel-coupled circuits of different organs (see Melander and Johansson 1968; Folkow and Neil 1971). In a recent study Gillespie and Rae (1972) showed that the extent of neurogenic response of isolated perfused arteries is related to their innervation density and wall to lumen ratio. In veins the isometric force response

TABLE II Sensitivity to exogenous NA  $ED_{50}$   $10^{-7}$  M

Blood vessel	Control	DMI $10^{-7}$ M	Control DMI $10^{-7}$ M	Propranolol $10^{-7}$ M + DMI
Pulmonary artery ( $n=8$ )	1.8 $\pm$ 0.37	1.1 $\pm$ 0.22	0.45 $\pm$ 0.05	0.53 $\pm$ 0.06
Ear artery ( $n=4$ )	4.9 $\pm$ 0.64	0.39 $\pm$ 0.11		
Small saphenous vein ( $n=4$ )	2.7 $\pm$ 0.0	0.94 $\pm$ 0.18	1.5 $\pm$ 0.53	1.7 $\pm$ 0.64

to nerve stimulation may be correlated primarily with the supply of adrenergic nerves (Bevan *et al* 1974 a b)

In the present experiments the amplitude of the maximum neurogenic responses relative to their own maximum responses to exogenous NA (Fig. 1 Table I) did not fall into artery and vein groupings. However the veins tended to respond relatively more than the arteries to stimulation at low impulse rates as reflected by steeper initial segments of the frequency response curves (Fig. 2 Table I)

In different blood vessels considerable variation has been found to occur in such factors as density and distribution of the terminal adrenergic plexus width of neuro-muscular separations mechanisms for transmitter elimination and properties of the effector tissue (for ref see Bevan and Su 1973). The present experiments were performed to make a comparison possible between frequency response relationships of some isolated arteries and veins. In the following the results will be discussed in relation to available information about their neuro-effector organization.

The pulmonary artery gave the lowest maximum neurogenic response. Furthermore a high impulse rate (14 Hz) was required to elicit half the maximum response. In rabbit elastic arteries very wide clefts prevail between the adrenergic plexus at the adventitia-media junction and the nearest smooth muscle cells (Verity and Bevan 1968). The deeper parts of the media become activated as the transmitter diffuses into succeeding muscle layers since myogenic spread of activity does not seem to occur (Bevan *et al* 1970). As a consequence the transmitter is distributed within a large fluid compartment and the mean effective concentration in the media is comparatively low (Bevan and Su 1974). The amplitude of the maximum isometric response to nerve stimulation and the steepness of the frequency response curve would be determined primarily by the mean NA concentration attained within the entire muscle layer during nerve activity at the different impulse rates. It may be that a failing transmitter output at high impulses rates (e.g. Brown and Gillespie 1957; Arpekar and Misra 1967; Davies and Withrington 1968; Haggendal *et al* 1970) at least in part sets the level of the maximal neurogenic response in some large vessels and causes the decreased responses found at supramaximal impulse rates.

In the rat portal vein a commonly used model for *in vitro* studies of spontaneously active vascular muscle the neuro-muscular separation (cf Boaz 1971) is more than one order of magnitude less than in the elastic arteries of the rabbit. There is evidence that a functional neuro-muscular junction exists in this vessel (see Ljung 1970). High but short lasting transmitter concentration peaks induce excitation of the muscle cells next to the nerve terminals from where the activity seems to be propagated myogenically throughout the media. The frequency response curve of the rat portal vein (Ljung 1970) included in Fig. 1 III was steep in the initial segment and showed one of the greatest response values to nerve stimulation. According to a quantitative model analysis of the neuroeffector function in the portal vein (Johansson *et al* 1972) the shape of the curve and the amplitude of the maximal neurogenic response relative to the exogenous NA response are greatly influenced by postjunctional mechanisms in that vessel.

The pattern of the neuro-effector function of the rat portal vein is essentially different from that which pertains to elastic arteries. However in spite of the two different pr



that appear to govern adrenergic vasomotor control of the rabbit pulmonary artery and the rat portal vein frequency response curves of similar configuration are obtained in the two vessels (Fig. 2).

It is likely that the rabbit elastic arteries and the rat portal vein respectively represent rather extreme variants in a spectrum of vascular neuro muscular arrangements (see Bevan and Su 1973, Ljung 1970). This assumption is supported by the present finding that the maximum neurogenic responses of all the muscular arteries except the proximal saphenous artery and of the peripheral veins ranged intermediately between the extremes of these two vessels. In the proximal saphenous artery the adrenergic terminal nerve structures form a dense network at the adventitia medial junction and in addition they do enter the outer media whereas the distal section of the same artery receives only a two-dimensional nerve plexus (Bevan and Purdy 1973). The maximum neurogenic response of this vessel was significantly smaller and the frequency response curve was less steep than its proximal counterpart (Fig. 1, 2, Table I). It is suggested that the great maximal neurogenic response and the high sensitivity to transmural nerve stimulation in the low frequency range found in the preparation with medial innervation (Fig. 1, 2, Table I) is related to the shorter distances required for transmitter diffusion and/or myogenic spread of neurogenically induced activity.

The small saphenous vein also has adrenergic innervation inside the medial layer (Bevan *et al.* 1974 b). This thin walled cutaneous vein often showed phasic activity superimposed upon the tonic nerve induced responses which demonstrates the existence of a presumably myogenic coordinating mechanism (*cf.* Johansson and Ljung 1967). Since longitudinal propagation of activity occurs in small peripheral arteries such as the posterior tibial artery (Bevan and Ljung 1974) it seems likely that myogenic mechanisms may contribute to the recruitment and coordination of medial cells in the response of small arteries and veins.

The maximum neurogenic response of the parietal branch of the internal iliac vein was comparable to that of the small saphenous vein. The former vessel is supplied with a two-dimensional plexus (Bevan *et al.* 1974 b) the density and NA uptake capacity of which is slightly less than that of the small saphenous vein. Tissue sensitivity to exogenous NA, extra neuronal NA uptake and activity of NA catabolizing enzymes seem comparable in the two vessels (Bevan *et al.* 1974 a). The observed difference in steepness of the frequency response relationships of these two veins (Fig. 2, Table I) thus might be the consequence of differences in the distribution of the nerve terminals.

In the present experiment the maximum neurogenic response of the ear artery of the rabbit which receives a heavy longitudinal innervation was 75 per cent of the NA response and thus was comparable to the small saphenous artery and the two peripheral veins (Table II). The sensitivity to exogenous NA was greater in the ear artery than in both the pulmonary artery and in the small saphenous vein after blockade of the neural uptake mechanism (Table II). This is in accordance with a previously noted tendency for the NA  $ED_{50}$  values of various venous effector tissues to be inversely related to the density of innervation (Bevan *et al.* 1974 b). Thus it appears that vascular smooth muscle from the same species but from different blood vessels exhibits variation in NA sensitivity which may be correlated with the dimensions of the vasomotor nerve supply. The significance of this finding awaits further experi-

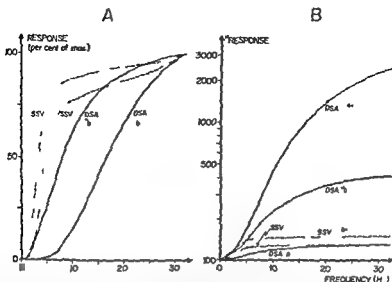


Fig 4 Frequency response curves of small saphenous vein (SSV) and distal saphenous artery (DSA). In A curves a and b correspond to the curves in Fig. 2. a and b<sup>4</sup> represent the relationship for the square and fourth power responses respectively when expressed as a percentage of max response. In B response in absolute terms have been expressed as a percentage of control as using that maximal activation of the muscle corresponds to shortening by 30 per cent. Note the pronounced change in resistance expected to occur in the artery ( $r_1^4$ ) assuming a wall to lumen ratio of 1/5 as compared to the capacitance response (a) in the vein for the same degree of smooth muscle shortening.

mental analysis. The dense adrenergic plexus of the ear artery also explains the pronounced prejunctional supersensitivity (Table II) obtained when exogenous NA is applied *via* the adventitial surface after elimination of the neuronal NA uptake mechanism (de la Lande Frewin and Waterson 1967). In the present experiments DMI reduced the ED<sub>50</sub> value by a factor of 13 in the ear artery as opposed to a value of 3 and 2 in the small saphenous vein and the pulmonary artery respectively (Table II).

The frequency response curve of the ear artery ranged in between those of the pulmonary artery and the rat portal vein with regard to steepness and maximal response (Table I) but was comparable to most of the curves for the peripheral vessels studied. Thus it can be concluded that in spite of an extraordinarily heavy terminal nerve plexus and a comparatively high effector sensitivity to exogenous NA the frequency response characteristics in the ear artery are similar to those in other vessels of corresponding calibre.

On the whole the present results demonstrate that the general configuration of curves relating isometric force responses to nerve stimulation impulse frequency is similar for a variety of isolated rabbit vessels. However differences do exist particularly in the steepness of the initial part of the curve. The presence of nerve terminals within the media and myogenic propagating mechanisms are apparently of importance.

The postganglionic sympathetic fibres to blood vessels normally discharge at low rates and even under intense reflex activation tonic impulse rates rarely exceed 6–8 Hz (Kow 1952, Kendrick Öberg and Wennergren 1972). Thus the important part of the frequency response curve must be the initial segment, where the major dif-

vessels also seem to occur (cf Fig 1) *In vivo* cardiovascular responses will not be linearly related to effector response and some consequences of this fact are illustrated in Fig 4. The frequency response curves of the small saphenous vein (curve a) and the distal saphenous artery (curve b) from Fig 2 are taken to represent smooth muscle activation. If the muscle had also been allowed to shorten like in the physiological situation then presumably the curves would have been steeper (cf Rosenblueth 1932). The curves of these two cutaneous vessels from the same section of the rabbit's hind limb actually displayed the greatest and the smallest curve steepness respectively. *In vivo* the functional response of the vein would be to reduce its content of blood, a parameter proportional to the square of the smooth muscle shortening whereas the resistance function of an artery would be related to the fourth power of the smooth muscle contraction. The curve relating the square ( $a^2$ ) and the fourth power ( $b^4$ ) of the response values to impulse frequency are displaced to the right to different extents.

Mellander (1960) found frequency response relationships *in vivo* for capacitance and resistance vessels in the hind quarter preparation which clearly resembled curve  $a^2$  and  $b^4$  (Fig 4 A) respectively when studied under conditions of constant arterial inflow pressure and venous outflow pressure. However in another report Browse, Lorenz and Shepherd (1966) found identical characteristics for the corresponding two frequency response relationships when capacitance responses of the dog's occluded hind limb were compared to those of the resistance vessels perfused at constant flow. The differences between the results of these two studies have been attributed to methodological factors (Mellander and Johansson 1969). The variation in frequency response characteristics of isolated arteries and veins, albeit from larger vessels, found in the present study in conjunction with the above mentioned influence of different mathematical power functions renders it less likely that a similar frequency response relationships would prevail in the resistance and capacitance sections of a vascular bed *in vivo*.

Mellander (1960) stressed that the vasoconstrictor effects on the venous compared to the arterial sections were more pronounced only when frequency response relationships were expressed as a percentage of the maximum neurogenic response. The present results could be utilized to compute approximate capacitance and resistance responses of the small saphenous vein and the distal saphenous artery if it is assumed that the isometric force responses could be taken to represent smooth muscle shortening. If it is further assumed that the maximal response of both tissues correspond to a decrease by 10 per cent of the outer wall radius the frequency response curves would be represented by a and b in Fig 4 B. The capacitance response of the small saphenous vein would be related to the change in cross-sectional area (curve a) and the change in resistance of the artery would accordingly be inversely related to the fourth power of the change in vascular radius (curve b).

The wall to lumen ratio of the vessels is known to be of great importance in determining hemodynamic effects. It is as well known that the wall thickness of the distended small saphenous vein can be disregarded but that the wall thickness to lumen radius is 1/4 in the distal saphenous artery (cf Bevan and Johnson 1973). As a result the "resistance" curve (b) which relates the change in the fourth power of the internal radius to impulse rate becomes greatly augmented.

In spite of the approximations involved the calculated maximal values which thus correspond to a reduction of venous blood content by 50 per cent and an increase in arterial flow resistance by a factor of 10-20 are of the order of magnitude found *in vivo* (cf Melander 1960). It is thus evident that seemingly small differences in the characteristics of frequency response relationships of isolated blood vessels due to variation in neuro-effector properties can be of great hemodynamic significance in view of the augmentation of effects derived from geometrical factors.

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## Stimulation of Adrenergic Nerve Fibres to the Urinary Bladder of the Rat

By

MATS ELMER

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### Abstract

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The contraction of the rat detrusor muscle caused by electrical stimulation of the hypogastric or pelvic nerves was followed by relaxation when the nerves were stimulated for a short period. The relaxation was more pronounced when the initial contraction was reduced by atropine. It was found to be mediated by adrenergic fibres acting on inhibitory  $\beta$  receptors. Stimulation of the hypogastric or pelvic nerves at high frequencies increased the contractile response probably via adrenergic fibres acting excitatory  $\alpha$  receptors.

**Key words:** Urinary bladder nerve stimulation, adrenoceptors

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In pharmacological experiments the presence of both excitatory  $\alpha$  adrenoceptors and inhibitory  $\beta$ -receptors in the detrusor muscle of the rat has been shown (Elmer 1974 a) and histochemically adrenergic as well as cholinergic nerve fibres innervating the bladder have been detected in both the hypogastric and pelvic nerves (Alm and Elmer 1975).

In the present investigation the bladder responses to stimulation of adrenergic fibres in the hypogastric and pelvic nerves were studied.

### Methods

1 male albino rats of the Wistar strain weighing about 150 g were used.

The rats were anesthetized with chloralose (100 mg/kg) given through a cannula in a femoral vein after induction with ether. The bladder was exposed and the ureters were ligated. A glass cannula was inserted into the bladder through an incision in the urethra. The bladder was filled with 0.5 ml of physiological saline and the pressure developed by the detrusor muscle was recorded by means of a transducer and a polygraph.

The hypogastric nerves were cut distal to the hypogastric ganglia and the distal ends were stimulated jointly using a bipolar electrode. Each pelvic nerve was stimulated after section proximal to the pelvic plexus, which in the male rat forms a distinct ganglion located on the lateral surface of the prostate gland (Langworthy 1965). 3 Grass stimulators supplied with stimulus isolation units giving rectangular pulses with a duration of 1 ms, a frequency of 0.1-200 Hz and of supramaximal intensity (10 V) were used.

mm Hg 1mm

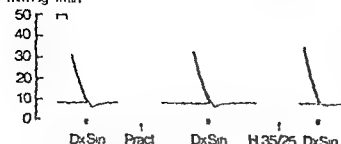


Fig. 1 Pressure responses of the rat urinary bladder to electrical stimulation of the pelvic nerves bilaterally at 0 Hz (Dx Sin) after the injection of atropine 1 mg/kg and dihydroergotamine 1 mg/kg. Pract: the  $\beta_1$  blocking agent practolol 10 mg/kg, H 35/25 the  $\beta_2$  blocking agent H 35/25 1 mg/kg.

For statistical evaluation of the data Student's *t* test for paired samples was used. The 0.05 level of probability was accepted as significant.

**Drugs.** The substances used were hexamethonium bromide, atropine sulphate, guanethidine bisulphate, dihydroergotamine methansulphonate, propranolol hydrochloride, practolol and H 35/25 (1-isopropyl amino-1-(4-methylphenyl) ethanol). The drugs were injected through the cannula in the femoral vein.

## Results

Electrical stimulation of the hypogastric or pelvic nerves at 20 Hz for a period of 15 s caused contraction of the detrusor muscle increasing the intravesical pressure from a resting pressure of about 10 mm Hg. When the nerves were stimulated for 5 s the contraction was followed by relaxation in 5 out of 9 rats. After the injection of atropine 0.1–1 mg/kg, the contraction of the bladder was reduced by 60% and was always followed by relaxation when the nerves were stimulated for a period of 5 s (Fig. 1). When the stimulation period was longer the relaxation was probably masked by predominant contraction in spite of the fact that the contractile response was reduced after atropine. Maximal relaxation was obtained with stimulation frequencies between 1 and 20 Hz decreasing the intravesical pressure by 0.5–4 mm Hg. The inhibitory response to stimulation of the pelvic nerves at 20 Hz was  $1.8 \pm 0.4$  mm Hg (mean  $\pm$  S.E.,  $n=9$ ) and the hypogastric nerves  $1.0 \pm 0.3$  mm Hg ( $n=9$ ).

The inhibitory responses were not affected by previous injection of dihydroergotamine 2 mg/kg or the  $\beta$  adrenoceptor blocking agent practolol 10 mg/kg but were totally abolished by guanethidine 1 mg/kg, propranolol 2–4 mg/kg or the  $\beta_2$ -blocking agent H 35/25 1 mg/kg (Fig. 1).

The contraction of the detrusor muscle caused by stimulation of the hypogastric nerve reached a maximum at stimulation frequencies of 15–20 Hz. At 30 Hz the response was

TABLE 1 Pressure responses in mm Hg of the rat urinary bladder to electrical stimulation of lateral (Lx) or pelvic (Px) nerves bilaterally (Lx or Px) and the pelvic nerves bilaterally (Dx or Sin). Values are mean  $\pm$  S.E.,  $n$  = number of experiments,  $p$  = significance at paired comparisons.

	Lx	Dx or Sin	Dx Sin
20 Hz	$2.1 \pm 0.5$	$18.9 \pm 6$	$51.6 \pm 8$
50–100 Hz	$5.1 \pm 1.5$	$41.8 \pm 7$	$61.9 \pm 9$
$n$	8	14	7
$p$	0.05	0.001	<0.1

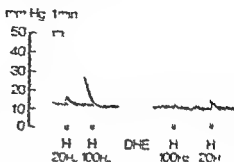


Fig. 2. Pressure responses of the rat urinary bladder to electrical stimulation of the hypogastric nerves (H) before and after the injection of dihydroergotamine (DHE).

somewhat lower but when the nerves were stimulated at higher frequencies the contractile response increased again, reaching another maximum which was 1–10 mm Hg higher at stimulation frequencies of 40–100 Hz. When the pelvic nerves were stimulated unilaterally using increasing frequencies the responses reached a plateau at 15 Hz and remained unchanged until the frequency was raised to 40 Hz. Then the response increased by 1–6 mm Hg to a maximum at 40–100 Hz. The response to bilateral stimulation of the pelvic nerves was not significantly changed by stimulation at these high frequencies (Table I).

The increased part of the responses caused by the high stimulation frequencies was not affected by previous injection of hexamethonium 10 mg/kg, atropine 1 mg/kg or propranolol 2 mg/kg but was totally abolished by guanethidine 1–3 mg/kg or dihydroergotamine 2 mg/kg (Fig. 2).

### Discussion

The contractile response of the rat bladder to stimulation of the pelvic nerves at 15–100 Hz is probably caused via cholinergic fibres (Carpenter and Rand 1964; Chisler 1967; Elmer 1973, 1975) and this seems also to be the case at stimulation of the hypogastric nerves in the guinea pig (Mantegazza and Naumzada 1967) and in the rat (Alm and Elmer 1975; Elmer 1975). These responses to hypogastric or pelvic stimulation in the rat are not affected by adrenoceptor blocking agents but reduced by about 60% after atropine and potentiated after eserine.

In dogs and cats some authors have found a relaxation of the bladder after an initial contraction when stimulating the hypogastric nerves (Griffiths 1959; Kuntz and Saccomanno 1944). The contraction in the cat is probably caused by activation of excitatory  $\alpha$ -adrenoceptors and the relaxation by inhibitory  $\beta$ -receptors (Edvardsson 1969). The relaxation is more protracted than the contractile response similar to the relaxation of the rat bladder found in this study when the hypogastric or pelvic nerves were stimulated for a short period and the contractile response was reduced by atropine.

The relaxation of the detrusor muscle found in the present investigation when the pelvic or hypogastric nerves were stimulated after atropine was abolished by the adrenergic blocking drug guanethidine indicating that the relaxation was caused by stimulation of the adrenergic fibres demonstrated histochemically in both nerves (Alm and Elmer 1975). The existence of two types of  $\beta$ -adrenoceptors was suggested by Lands *et al.* (1967) in the heart and small intestine and  $\beta_1$  in the bronchi blood vessels and uterus.



experiments have shown that the inhibitory  $\beta$  adrenoceptors of the rat urinary bladder belong to the type of receptors classified as  $\beta_2$ -receptors (Elmér 1974 b). The  $\beta_1$  receptor blocking compound practolol (Dunlop and Shanks 1968) did not affect the inhibitory bladder response to nerve stimulation in the present study when given in a dose which completely blocks the  $\beta_1$  receptors of the heart in the cat (Åblad *et al.* 1973). The relaxation of the bladder was however totally abolished by the non selective  $\beta$  adrenoceptor blocking agent propranolol or the  $\beta_2$ -blocking agent H 35/25 (Levy 1967, Levy and Wilkensfeld 1969, Carlsson *et al.* 1972, Johansson 1973) indicating that the inhibitory effect of adrenergic nerve stimulation is exerted by action on  $\beta_2$ -receptors.

The increased part of the contraction of the detrusor muscle obtained when one of the pelvic nerves or the hypogastric nerves were stimulated at high frequencies was not affected by hexamethonium, atropine or propranolol but was abolished by guanethidine or dihydroergotamine suggesting that postganglionic adrenergic fibres were stimulated acting on  $\alpha$  receptors. The presence of excitatory  $\alpha$  adrenoceptors in the detrusor muscle of the rat has been shown in pharmacological experiments (Elmér 1974 a). Garry and Gillespie (1955) found the same high stimulation frequency to be optimal for stimulation of the sympathetic nerves to the rabbit colon.

Hypothetically the explanation of the finding that the  $\alpha$  receptors were not activated at low stimulation frequencies but only at these high frequencies might be an overflow of transmitter from the adrenergic fibres shown to be present in the detrusor muscle partly around blood vessels (Ålm and Elmér 1975) activating  $\alpha$  receptors in muscle fibres not innervated by adrenergic nerves. The adrenergic innervation of the rat detrusor is sparse and the terminal fibres are not associated with individual muscle cells but only with large groups of muscle fibres (El Badawi and Schenk 1966, Ålm and Elmér 1975). The assumption that the excitatory  $\alpha$  receptors are activated when the amount of transmitter released is increased is in agreement with the results of Edge (1955) who obtained a bladder contraction in the cat by intraarterial injection of noradrenaline but relaxation after equivalent doses intravenously. Two weeks after denervation of the rat bladder the response to injected noradrenaline is changed from relaxation to contraction probably because of sensitization of  $\alpha$  receptors more than  $\beta$ -receptors (Elmér 1974 a). Six to ten weeks after parasympathetic denervation an outgrowth of new adrenergic terminals in the detrusor muscle of the cat is observed changing the normal inhibitory  $\beta$ -receptor response to hypogastric nerve stimulation to an excitatory  $\alpha$  receptor response (Sundin and Dahlström 1973). Thus existing but not utilized  $\alpha$  receptors might be supposed to be activated postulating that these receptors demand a higher concentration of noradrenaline than do the  $\beta$ -receptors.

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# Characterization of the Metabolism of Exogenous Cyclic AMP by Perfused Rat Heart and Incubated Prepubertal Rat Ovary

By

STEN ROSBERG, GUNNAR SELSTAM and OLLE ISAKSSON

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## Abstract

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In order to study the metabolism of extracellular 3',5' adenosine monophosphate (cAMP), rat hearts were perfused and prepubertal rat ovaries incubated with  $^3\text{H}$  and  $^{32}\text{P}$  labelled cAMP (0.05  $\mu\text{M}$ ). The rate of disappearance of cAMP from the medium was determined by  $^{65}\text{Zn}$  precipitation and degradation products of  $^3\text{H}$  and  $^{32}\text{P}$ -cAMP by paper chromatography. Both tissues degraded cAMP to 5' adenosine monophosphate (AMP), but the enzyme kinetic for this phosphodiesterase activity was different (apparent  $K_m$  value for the heart 3.95  $\mu\text{M}$  and for the ovary 0.2  $\mu\text{M}$ ). AMP was further degraded, hence also other labelled substances were found in the medium. An uptake of both  $^3\text{H}$  and  $^{32}\text{P}$  labelled substances into the heart and the ovary was noticed. Tissue extracts contained several labelled purines, but the amounts of labelled cAMP did not exceed expected amounts in the extracellular space. In the ovary the uptake of cAMP and AMP seemed to be low, since the uptake of labelled substances was reduced by high concentrations of unlabeled AMP or adenosine. The degradation of  $^{32}\text{P}$ -cAMP was much faster when AMP was present, strongly suggesting that the phosphodiesterase enzyme was acting extracellularly. In the heart added AMP was very rapidly degraded making it impossible to elucidate whether cAMP was degraded extracellularly or not. It is concluded that elimination of extracellular cAMP under physiological conditions can be due to degradation of cAMP by various tissues. At least for the ovary this phosphodiesterase enzyme acts extracellularly.

Cyclic 3',5' adenosine monophosphate (cAMP) is released from many tissues in response to hormonal stimulation, as has been shown in studies both *in vivo* and *in vitro* (Davoren and Suherland 1963, Estlin *et al.* 1972, Cramer and Lindl 1974, Franklin and Fox *et al.* 1971, Pentremann *et al.* 1973, Dole *et al.* 1973, Selstam *et al.* 1974). The magnitude of the release differs markedly between different types of tissues. In some tissues, e.g. the perfused liver and the incubated prepubertal rat ovary, a release of cAMP in response to low concentrations of hormones is measurable even without a detectable intracellular elevation of cAMP (Estlin *et al.* 1972, Rosberg *et al.* 1974). Under basal *in vivo* conditions the release of cAMP gives a relatively constant concentration of cAMP in the blood, although the turnover of cAMP in plasma is rapid (Chase and Aurbach 1967). Thus, a continuous supply of cAMP

to the plasma is counterbalanced by an elimination of cAMP from the plasma. Only a small part of the eliminated cAMP is excreted via the kidneys (Broadus *et al* 1970). The low *in vitro* hydrolysis of cAMP by the blood also suggests that blood phosphodiesterases play only a minor role in the elimination of cAMP (Hemington *et al* 1973). It is however not known which tissues in the body that contribute to the major degradation of the extracellular cAMP. It has been shown for some tissues that exogenously added cAMP can be degraded *in vitro* (MacManus *et al* 1971 Szabo and Burke 1972 Woo and Manery 1973). Of importance in this respect is that many enzymes participating in nucleotide metabolism are localized to the plasma membrane. In fact it has been shown that one of the enzymes in nucleotide metabolism, 5 nucleotidase degrading 5 adenosine monophosphate (AMP) to adenosine is available only for extracellular substrate (De Pierre and Karnovsky 1974).

From studies with incubated frog muscles Woo and Manery (1973) have presented evidence that the degradation of exogenous cAMP takes place extracellularly and that this degradation is stimulated by insulin *in vitro*. Since it is known that some endocrine diseases are associated with altered extracellular levels of cAMP the characterization of the extracellular cAMP degradation system(s) deserves special attention. It is thus possible that hormones might directly regulate membrane localized enzymes in the nucleotide metabolism and thereby affect the levels of regulator substances like cyclic nucleotides with recognized importance for the cell metabolism. In a forthcoming study evidence will be presented that FSH *in vitro* stimulates membrane bound phosphodiesterase activity in the prepubertal rat ovary (Selstam and Rosberg 1975).

The aim of the present study was to see whether exogenous cAMP is degraded and to characterize such a degradation by enzyme kinetics and by determining degradation products by paper chromatography in two isolated but intact organs the perfused rat heart and the incubated prepubertal rat ovary. The release of cAMP during hormonal stimulation is much greater from the incubated prepubertal rat ovary (Selstam *et al* 1974) than from the perfused rat heart (Isaksson to be published) and it was considered of interest to compare these tissues. We have in this study measured the degradation of cAMP at a concentration of  $10^{-8}$  M or lower since estimated concentrations of cAMP in most extracellular fluids do not exceed these levels. Preliminary reports on part of this study have been published elsewhere (Rosberg *et al* 1974, Selstam *et al* 1975).

## Materials and Methods

## Chem als

The ( $^3\text{H}$ )-cAMP (100-2000 Ci/mol at experimental date 96-99 purity) and ( $^3\text{H}$ )-cAMP (75 Ci/mol 98-99 purity) were purchased from The Radiochemical Centre Amersham ( $^3\text{H}$ )-5' adenosine monophosphate (cAMP 35 Ci/mol 98-99 purity) was obtained from the New England Nuclear Co.

Radioactive substances except  $^{32}\text{P}$ -cAMP were purified by paper chromatography when less than 99% pure. All other chemicals including AMP, adenosine, adenine and theophylline were of analytical grade and purchased from Merck or Sigma Chemical Co.

## Exp. n. total proc. durres

**Preparation of hearts** Hearts from fed female Sprague-Dawley rats, weighing 140-160 g were used. The rats were anesthetized with ether and the hearts were rapidly excised and transferred to ice-chilled

The aorta was cannulated and retrograde perfusion with Krebs bicarbonate buffer at 37°C from a reservoir 70 cm above the heart was started immediately. After a 5 min washout period the hearts were perfused for 90 min as a modified Langendorff preparation with recirculating Krebs bicarbonate buffer with half the normal calcium concentration (1.25 mM) and containing glucose (7.5 mg/ml),  $^{32}\text{P}$ -cAMP (0.1–1.0  $\mu\text{M}$ ) and  $^3\text{H}$ -cAMP (0.1–1.0  $\mu\text{M}$ ). In some experiments  $^{14}\text{C}$  AMP (1 mM) was added. The recirculating volume was 100 ml at the start of the perfusion period and it was continuously gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  equilibrated with water. The temperature of the perfusate was maintained at 37°C and the perfusion pressure was 80 mm Hg. For further details concerning the heart perfusion technique see Isaksson (1971) and Morgan *et al.* (1961). At different time periods during the perfusion 0.1 ml aliquots of the perfusion medium were withdrawn in duplicates from the coronary effluent with a micropipette. To one aliquot  $\text{ZnSO}_4$  and  $\text{Ba}(\text{OH})_2$  were added for  $\text{Ba}/\text{Zn}$  precipitation (see below) and to the other water in equal amounts. The present sampling technique made it possible to follow the changes in the metabolism of exogenously added labelled cAMP and its degradation products in the perfusion medium with time. At the end of the perfusion period the hearts were frozen between blocks of aluminium cooled to the temperature of liquid  $\text{N}_2$ . The hearts were stored at  $-80^\circ\text{C}$  until analyzed.

**Incubation of ovaries.** Sprague-Dawley rats 23–24 days old weighing 45–49 g were used. They were starved for 18–24 h before the experiments. The rats were sacrificed by cervical fractures and the ovaries rapidly removed and placed in ice-chilled buffer. Each ovary was carefully trimmed free from extraneous tissues under a stereomicroscope, rinsed, blotted on filter paper, weighed on a torsion balance and placed in a "preincubation" flask. Only ovaries weighing between 6 and 9 mg were used. The preincubation medium consisted of 1 ml Krebs bicarbonate buffer with half the normal calcium concentration (1.25 mM) and containing glucose (1 mg/ml). The ovaries were preincubated for 60 min at 37°C under continuous shaking and with 95%  $\text{O}_2$ –5%  $\text{CO}_2$  as gas phase. The ovaries were then transferred to 15 ml glass tubes and incubated under the same conditions in 0.5 ml medium with the addition of  $^{32}\text{P}$ -cAMP (0.05–1.0  $\mu\text{M}$ ),  $^3\text{H}$ -cAMP (0.05–1.0  $\mu\text{M}$ ) and in some experiments AMP, adenosine, adenine or theophylline at a concentration of 1 mM. Incubation periods lasted for 60–120 min and at the end of the incubations the ovaries were immediately frozen in Frenzel chilled with solid carbon dioxide and stored at  $-80^\circ\text{C}$  until analyzed.

#### Treatment of tissues and media

A piece of the frozen left ventricle of the heart (15–40 mg) or one intact ovary was homogenized in 1.5 ml 5% trichloroacetic acid and centrifuged. The supernatant was extracted three times with ether, redissolved overnight and redissolved in 0.5 ml water. To 0.1 ml aliquots of these "tissue extracts" and also 10 media samples equal amounts of 0.15 M  $\text{ZnSO}_4$  and 0.15 M  $\text{Ba}(\text{OH})_2$  were added according to the method described by Krishna *et al.* (1968). After centrifugation (1000 g for 70 min) 10 ml of Insta Gel<sup>®</sup> (Packard) was added to 0.1 ml aliquots of the supernatant and the radioactivity was measured in a Packard Tricarb Scintillation Spectrometer (Model 330). To aliquots of non-precipitated tissue and media samples water was added in equal amounts and radioactivity was measured in the same way. The quenching was checked with external standards and was found to be equal for all samples within an experiment. Corrections were made for the  $\text{P}$ -decay. Krishna *et al.* (1968) reported that  $\text{ZnSO}_4$  and  $\text{Ba}(\text{OH})_2$  completely precipitated  $^{32}\text{P}$  cAMP and adenosine. In the present investigation less than 0.1% of labelled cAMP was found to be precipitated under the experimental conditions used. Adenosine was found to be precipitated by  $\text{ZnSO}_4$ . The following substances were found to be completely precipitated by  $\text{ZnSO}_4$ : adenosine triphosphate (ATP), 5'-adenosine diphosphate (ADP), AMP, inorganic phosphate, adenosine, hypoxanthine and cytosine. 3',5'-guanosine monophosphate (cGMP). The following experiments were carried out at 0–4°C.

#### Paper chromatography

Aliquots of 0.05 ml were applied as narrow bands on Whatman No. 1 paper (4 cm  $\times$  46 cm). Descending chromatograms were run in 0.1 M ammonium acetate (pH 7.15) at a flow rate of 1.5 ml/min. The chromatograms were developed and of these purines the positions of cAMP and cGMP were determined. The chromatograms were then cut into 2 cm segments and separately placed in 10 ml of 10% trichloroacetic acid. The radioactivity was counted in a Beckman LS 5000TD. CPM values were corrected for quenching and decay.

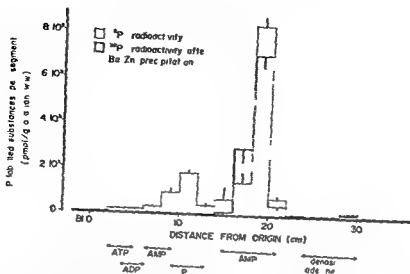


Fig. 1 Distribution of  $^{32}\text{P}$  radioactivity in ovarian incubates on medium determined by paper chromatography. Aliquots of non-treated and Ba-Zn precipitated media from ovaries incubated for 2 h in  $0.25 \mu\text{M}$   $^{32}\text{P}$ -cAMP were chromatographed on Whatman paper No. 1. Descending 36 cm chromatograms were run for 1 h with 96% ethanol : 4 M ammonia : 2 M acetic acid (100 : 19 : 21) pH 7.15. The chromatograms were cut in 1 cm segments which were counted in a liquid scintillation spectrometer. The amounts of labelled substances per segment were calculated as described in Methods. Each histogram represents mean values of segments from 3 chromatograms. S.E. is indicated by a vertical bar. The positions of chromatographed unlabelled ATP, ADP, AMP, cAMP, adenosine and adenine as visualized by ultra-violet light are shown in the lower part of the figure.

radioactivity for decay. The relative yield in the paper chromatograms (total CPM for an 0.05  $\mu\text{l}$  aliquot applied to a segment CPM for an equal aliquot diluted directly in 12 ml of Insti-Gel) was for  $^{32}\text{P}$  radioactivity 99%,  $^3\text{H}$  radioactivity 73% and for  $^3\text{H}$  radioactivity 15%. A typical chromatogram with the distribution of  $^{32}\text{P}$  labelled substances is shown in Fig. 1.

#### Calculations and statistical procedures

Degradation of  $^{32}\text{P}$ -cAMP and uptake of  $^3\text{H}$  and  $^{32}\text{P}$  adenosine. The rate of  $^{32}\text{P}$ -cAMP degradation was determined by measuring the disappearance rate of radioactivity from Ba-Zn-precipitated perfusate and incubation media. The uptake of  $^3\text{H}$  and  $^{32}\text{P}$  labelled substances was measured as disappearance of radioactivity from non-precipitated media. Degradation and uptake were corrected for dilution of radioactivity into the extra-cellular space as determined by sucrose (Hjalmarsson and Isaksson 1972; Nilsson and Sehtman 1973). The degradation and uptake of labelled substances based on the disappearance of radioactivity from the medium was calculated using the following formula:

$$[\text{CPM}_{t_0} - \text{CPM}_{t_1}] / ((V + ec)/V) \cdot S = V \cdot (\text{CPM}_{t_0} \cdot w)$$

where  $\text{CPM}_{t_0}$  = radioactivity in non-precipitated or Ba-Zn-precipitated media samples at the indicated time;  $V$  = perfusion or incubation volume;  $ec$  = extra-cellular volume;  $S$  = concentration of cAMP at zero time;  $w$  = tissue wet weight.

Distribution of labelled substances in chromatograms. The amounts of labelled substances in segments from a chromatogram were calculated by multiplying the percentage of  $^3\text{H}$  and  $^{32}\text{P}$  radioactivity in the segments with the calculated amounts of labelled substances in the chromatographed medium or tissue. The distribution of labelled substances in the tissue was corrected for radioactivity in the extra-cellular space by assuming an equal distribution of labelled substances in the medium and in the extra-cellular space.

Statistical procedure. Mean values are given  $\pm$  S.E. Comparisons were made by Student's  $t$ -test. A  $p$ -value of 0.05 or less was considered significant in this study.

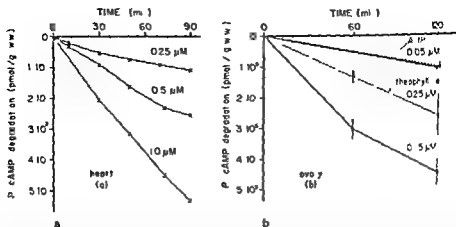


Fig. 2. Degradation of extracellular  $^{32}\text{P}$ -cAMP by rat heart (a) and prepubertal rat ovary (b). Rat hearts were perfused with 10 ml Krebs bicarbonate buffer containing glucose (5 mg/ml) and  $^{32}\text{P}$ -cAMP (0.5–1.0  $\mu\text{M}$ ). Aliquots of the perfusion medium were taken during the perfusion.  $^{32}\text{P}$ -cAMP contents in the media were determined by measuring the radioactivity after Ba-Zn precipitation. The values for each concentration presented are from individual hearts. Prepubertal rat ovaries were incubated in 0.5 ml Krebs bicarbonate buffer containing glucose (1 mg/ml) 0.05  $\mu\text{M}$   $^{32}\text{P}$ -cAMP with or without unlabelled AMP (1 mM) and 0.75  $\mu\text{M}$   $^{32}\text{P}$ -cAMP with or without theophylline (1 mM). Ovaries were incubated for the times indicated and  $^{32}\text{P}$ -cAMP degradation in the media was determined as described above. Each point represents the mean value of 5 ovaries. S.E. is indicated by a vertical bar.

## Results

It will be seen from Fig. 2 that both the heart and the ovary degraded  $^{32}\text{P}$ -cAMP in the perfusion and incubation media respectively. The rate of degradation of  $^{32}\text{P}$ -cAMP was found to decrease somewhat at the end of the perfusion and incubation periods, probably due to the decreasing concentrations of labelled cAMP in the media with time. On increasing the initial concentration of cAMP in the medium the rate of degradation of cAMP was increased (Fig. 2a and b). The rate of degradation was decreased approximately 40% when theophylline at a concentration of 1 mM was added to the ovarian incubation media when the initial concentration was 0.25  $\mu\text{M}$  (Fig. 2b).

In order to study enzyme kinetics the degradation rates for different concentrations of  $^{32}\text{P}$ -cAMP were determined. Rat hearts were perfused with  $^{32}\text{P}$ -cAMP at different concentrations (0.1–1.0  $\mu\text{M}$ ) and samples from the perfusion medium were taken at 0, 10, 30, 60 and 90 min after the start of perfusion. Since fairly straight lines were obtained when the velocities for the degradation of  $^{32}\text{P}$ -cAMP between the sampling times were plotted versus time, initial velocities for the different concentrations were achieved by extrapolation to zero time. In Fig. 3 inverted values of the calculated initial velocities are plotted versus inverted substrate concentrations (Lineweaver and Burk 1934). A straight line calculated with the method of least squares (regression coefficient  $r=0.99$ ) gives an apparent  $K_m$  of 1.95  $\mu\text{M}$  and an apparent  $V_m$  of 315 pmol/g w.w./min for this phosphodiesterase activity of the rat heart. Fig. 4 demonstrates a Lineweaver-Burk plot for phosphodiesterase activity of the incubated prepubertal rat ovary. Ovaries were incubated for 120 min with  $^{32}\text{P}$ -cAMP at different concentrations (0.05–1.0  $\mu\text{M}$ ) and the velocity for each concentration was calculated.

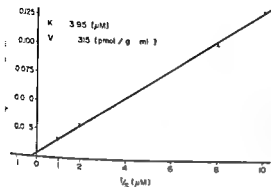


Fig 3 Lineweaver Burk plot of phosphodiesterase activity in perfused rat heart. Rat hearts were perfused with different  $^{32}\text{P}$  cAMP concentrations (0.1–1.0  $\mu\text{M}$ ) as described in Methods. Aliquots were taken from the media at different times (10–90 min) and  $^{32}\text{P}$ -cAMP content was determined by Ba-Zn precipitation. The calculated initial velocities presented in the figure were estimated by extrapolating the velocities between consecutive sampling times for each concentration to zero time. The regression line was calculated by the method of least squares with a regression coefficient of 0.996 based on observations from 6 individual hearts.

culated. For the concentration range of 0.025–0.25  $\mu\text{M}$  a straight line was calculated by the method of least squares giving an apparent  $K_m$  of 0.20  $\mu\text{M}$  and a  $V_{\text{max}}$  of 49 pmol/g wet wt  $\times$  min. The two highest concentrations tested (0.5 and 1.0  $\mu\text{M}$ ) were not used in these calculations of kinetics since a deviation from linearity for these concentrations was a consistent finding in several experiments. In preliminary experiments indication of a phosphodiesterase enzyme with a higher  $K_m$  value has been found which might explain the deviation from linearity when the concentration of cAMP was increased above 0.25  $\mu\text{M}$ .

Both the heart and the ovary accumulated  $^3\text{H}$  and  $^{32}\text{P}$  labelled substances. In the heart the uptake was fairly equal for both labelled substances (Fig 5a) while in the ovary the uptake of  $^3\text{H}$  labelled substances was much more pronounced (Fig 5b). The rate of uptake of  $^3\text{H}$  labelled substances was for both tissues found to be fairly linear with time and more pronounced with increasing  $^3\text{H}$  cAMP concentrations (Fig 5). In the above mentioned experiments the uptake was calculated on the disappearance of radioactivity from the medium. When the uptake of labelled substances was calculated on the radioactivity in tissue extract of the heart with correction for the extracellular space the uptake after 90 min of perfusion with 0.5  $\mu\text{M}$  labelled cAMP was 604 and 510 pmol labelled substances/g wet weight for H and  $^{32}\text{P}$  radioactivity respectively (cf Fig 5a). This difference in results between the two methods of determining uptake of labelled substances is probably due to

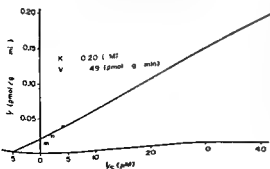


Fig 4 Lineweaver Burk plot of phosphodiesterase activity in incubated prepubertal rat ovary. Prepubertal rat ovaries were incubated with different  $^{32}\text{P}$ -cAMP concentrations (0.05–1.0  $\mu\text{M}$ ) for 120 min as described in Methods. At the end of the incubation period the  $^{32}\text{P}$ -cAMP content of the incubation media was determined by Ba-Zn precipitation. The regression line was calculated by the method of least squares and the regression coefficient was found to be 0.984 when the values for the two highest concentrations (0.5, 1.0  $\mu\text{M}$ ) were excluded. Each point represents the mean of 3 ovaries.



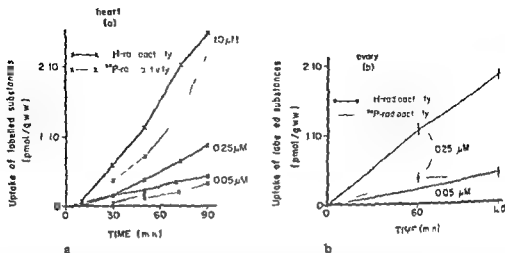
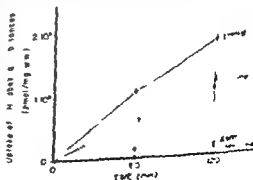


Fig. 5 Uptake of  $^3\text{H}$  and  $^{32}\text{P}$  labelled substances by rat heart (a) and prepubertal rat ovary (b). Rat hearts were perfused with 10 ml Krebs bicarbonate buffer containing glucose (2.5 mg/ml) and  $^3\text{H}$  and  $^{32}\text{P}$ -cAMP (0.25–1.0  $\mu\text{M}$ ). Aliquots of the perfusion medium were taken during the perfusion. Prepubertal rat ovaries were incubated in 0.5 ml Krebs bicarbonate buffer containing glucose (1 mg/ml) and  $^3\text{H}$  and  $^{32}\text{P}$ -cAMP (0.05 and 0.25  $\mu\text{M}$ ). Uptake of  $^3\text{H}$  and  $^{32}\text{P}$  radioactivity was determined by measuring the disappearance of  $^3\text{H}$  and  $^{32}\text{P}$  radioactivity from the medium as described in Methods. For the ovary each point represents the mean value of 5 ovaries. S.E. is indicated by a vertical bar.

loss of radioactivity during weighing and freezing of the heart. A similar difference although smaller was also noticed for the ovary.

To determine whether or not the degradation of cAMP to AMP occurred extracellularly, unlabelled AMP was added to the ovarian incubation media at a concentration of 1 mM. As may be seen from Fig. 6, AMP effectively inhibited the uptake of  $^3\text{H}$  labelled substances, suggesting that cAMP was degraded extracellularly (Fig. 6). The degradation of  $^{32}\text{P}$ -cAMP was unchanged in the presence of AMP (Fig. 2b). AMP seemed also to be degraded extracellularly since unlabelled adenosine (1 mM) effectively inhibited the uptake of  $^3\text{H}$  labelled substances. In contrast to AMP and adenosine, unlabelled adenine (1 mM) inhibited the uptake only moderately. In order to see whether the phosphodiesterase activity was bound to the tissue or could be found in the medium, ovaries were first incubated for 4 hours

Fig. 6. Uptake of  $^3\text{H}$  labelled substances by prepubertal rat ovary with and without added AMP, adenosine, adenine or theophylline. Ovaries were incubated in 0.5 ml Krebs bicarbonate buffer containing glucose (1 mg/ml) and  $^3\text{H}$ -cAMP (0.5  $\mu\text{M}$ ) and incubated for the periods indicated. The test substances were added at a concentration of 1 mM. Uptake of  $^3\text{H}$  radioactivity was determined by measuring the disappearance of  $^3\text{H}$  radioactivity from the medium as described in Methods. Each value represents the mean of 5 ovaries. S.E. is indicated by a vertical bar.



without labelled cAMP. Ovaries were then removed from the media and  $^3\text{H}$ -cAMP was added to the medium (final concentration  $1\ \mu\text{M}$ ) and the media were incubated for an additional 2 h. The  $^3\text{H}$ -cAMP degradation in these media was less than 5% compared to media containing ovaries. It was not possible with the present procedures to find out whether the degradation of labelled cAMP in the medium by the heart occurred extracellularly since it was found with chromatographic analysis that  $1\ \text{mM}$   $^3\text{H}$ -cAMP was completely degraded after only 10 min of perfusion.

Distribution of radioactivity as determined by paper chromatography of perfusate and tissue extracts from rat hearts perfused with  $0.5\ \mu\text{M}$   $^3\text{H}$  and  $^3\text{H}$ -cAMP are shown in Table I. The amounts of  $^3\text{H}$  and  $^3\text{H}$ -cAMP found in the tissue after 90 min of perfusion corresponded very closely to the expected amounts in the extracellular space demonstrating that the concentration of intracellularly located labelled cAMP must have been close to zero. The main part of  $^3\text{H}$  labelled substances in the tissue extract was found in the adenosine adenine region of the chromatograms (Table I). Small amounts of  $^3\text{H}$  labelled substances were found in the ATP ADP region and in the AMP region.  $^3\text{P}$  labelled substances were found in the ATP ADP AMP and  $\text{P}_i$  regions.

Aliquots of the perfusion medium were withdrawn at different periods of time and chromatographed to study the change in the amounts of labelled cAMP and its degradation products (Table I). Only small amounts of  $^3\text{H}$  labelled substances were found apart from  $^3\text{H}$ -cAMP. In the adenosine adenine region a fairly constant amount was seen throughout the perfusion period and seemed largely to consist of adenosine since approximately 50% remained after Ba-Zn precipitation.  $^3\text{P}$  labelled substances were found in all regions indicated in Table I. In the AMP region a fairly constant amount was found during the perfusion period while in the ATP ADP region and to an even greater extent in the  $\text{P}_i$  region an accumulation in the perfusion medium was seen with time. An impurity of the  $^3\text{P}$ -cAMP preparation (1-2%) found in the adenosine adenine region of non perfused medium (0 min Table I) remained in the medium during perfusion. The pattern of distribution of  $^3\text{H}$  and  $^3\text{P}$  labelled products with time was the same for all concentrations of cAMP ( $0.1$ – $10\ \mu\text{M}$ ).

At all times studied a difference between the amounts of  $^3\text{H}$  and  $^3\text{P}$ -cAMP was seen in the rat heart perfusion medium (Table I). The observed degradation rate of  $^3\text{P}$ -cAMP was approximately twice as high as that of  $^3\text{H}$ -cAMP suggesting recirculation of tritiated compounds to  $^3\text{H}$ -cAMP during the perfusion. This difference between  $^3\text{H}$  and  $^3\text{P}$ -cAMP degradation was seen for all concentrations of cAMP ( $0.1$ – $10\ \mu\text{M}$ ) tested.

Table II demonstrates the pattern of degradation products in ovarian tissue extracts and incubation media after incubation periods of 60 and 120 min in the presence of  $0.25\ \mu\text{M}$   $^3\text{H}$  and  $^3\text{P}$ -cAMP. In contrast to the heart the rate of disappearance of  $^3\text{H}$  and  $^3\text{P}$ -cAMP was similar in the ovarian incubation media.  $^3\text{H}$  and  $^3\text{P}$  labelled substances in the tissue were found in the ATP ADP AMP and adenosine adenine regions. Small amounts of  $^3\text{P}$  labelled substances were found in the ATP ADP AMP and  $\text{P}_i$  regions. The degradation products in the medium from  $^3\text{H}$ -cAMP were found in the AMP region and to a greater extent in the adenosine adenine region of the chromatograms. In the latter region a proximately 2/3 was precipitated by Ba-Zn precipitation suggesting that aden



**Test II** Degradation of labelled cAMP in the medium and distribution of radioactivity in incubation medium and ovarian tissue determined by paper chromatography. Prepubertal rat ovaries were incubated for 60 and 120 min in 0.5 ml Krebs bicarbonate buffer containing glucose (1 mg/ml) and  $^3\text{H}$  and  $^{32}\text{P}$ -cAMP ( $0.25 \mu\text{M}$ ). After the incubation periods samples of the incubation media and tissue extracts were chromatographed before and after Ba-Zn precipitation. The table shows the degradation of cAMP in the medium and the distribution of radioactivity in medium and tissue. The amount of radioactivity in the tissue was calculated from the disappearance of radioactivity in the medium at the end of the incubation period. The distribution of radioactivity in the tissue has been corrected for amounts of radioactivity in the extracellular space of the ovary (30% of the wet weight) assuming the concentration of labelled products in the extra-cellular space to be the same as in the medium. Values presented represent means  $\pm$  S.E. of 5 ovaries.

	cAMP-degradation in the medium <sup>a</sup> (pmol labelled cAMP/g ovarian wet weight) $\times 10^{-3}$		Amounts of labelled substances in media and tissues (pmol labelled substances/g ovarian wet weight) $\times 10^{-3}$						
	Unprecipitated	Ba-Zn precipitated	Unprecipitated					Ba-Zn precipitated	
			0-6 <sup>b</sup> ATP ADP	6-10 AMP	10-14 P <sub>i</sub>	16- $\infty$ cAMP	4-30 Adenosine adenine	4-30 Adenosine	
<b><math>^3\text{H}</math>-radioactivity</b>									
Medium, incubation time (min)									
60	4.71 $\pm$ 0.70	3.65 $\pm$ 0.51	N D	0.6 $\pm$ 0.13	N D	— <sup>d</sup>	2.74 $\pm$ 0.46	0.94 $\pm$ 0.15	
120	5.48 $\pm$ 0.46	4.89 $\pm$ 0.43	N D	0.75 $\pm$ 0.25	N D	— <sup>d</sup>	2.71 $\pm$ 0.23	0.99 $\pm$ 0.15	
Tissue, incubation time (min)									
60	—	—	0.47 $\pm$ 0.05	0.14 $\pm$ 0.0	N D	N D	0.33 $\pm$ 0.04	N D	
120	—	—	0.03 $\pm$ 0.18	0.58 $\pm$ 0.07	N D	N D	0.80 $\pm$ 0.09	N D	
<b><math>^{32}\text{P}</math>-radioactivity</b>									
Medium, incubation time (min)									
60	3.86 $\pm$ 0.73	3.11 $\pm$ 0.47	0.33 $\pm$ 0.05	0.68 $\pm$ 0.13	0.34 $\pm$ 0.38	— <sup>d</sup>	0.35 $\pm$ 0.03	0.29 $\pm$ 0.0	
120	5.48 $\pm$ 0.70	4.78 $\pm$ 0.46	0.39 $\pm$ 0.03	0.96 $\pm$ 0.15	3.43 $\pm$ 0.50	— <sup>d</sup>	0.33 $\pm$ 0.0	0.3 $\pm$ 0.0	
Tissue, incubation time (min)									
60	—	—	0.1 $\pm$ 0.0	0.04 $\pm$ 0.01	0.06 $\pm$ 0.01	N D	N D	N D	
120	—	—	0.1 $\pm$ 0.04	0.01 $\pm$ 0.01	0.07 $\pm$ 0.03	N D	N D	N D	

<sup>a</sup> cAMP degradation was measured as initial radioactivity minus 16- $\infty$  cm parts of chromatograms.

<sup>b</sup> Regions of chromatograms (cm from origin) with the positions of chromatographed unlabelled purines and the position of phosphate.

<sup>c</sup> Not Detectable. These values were not significantly different from zero.

<sup>d</sup> Not shown due to differences in ovarian weight.

and Burke 1972) and rat thymic lymphocytes (MacManus *et al.* 1971) also degrade extracellular cAMP. These results thus imply that several tissues participate in the metabolism of extracellular cAMP and probably can account for the fact that the main part of cAMP in the plasma is eliminated by factors other than renal clearance. In the present study the elimination of cAMP by both the heart and the ovary obeyed enzyme kinetics. The apparent  $K_m$  value for the heart was found to be  $3.95 \mu\text{M}$ .  $K_m$  values of  $0.8 \mu\text{M}$  and  $3.85 \mu\text{M}$  have been reported for phosphodiesterase activities in homogenates and purified enzyme preparations respectively from the heart (Beavo *et al.* 1970; Thompson and App

In these as well as in other studies (Püsch 1971, Goren and Rosen 1971, Hrapchak and Rasmussen 1972) phosphodiesterase activities with  $K_m$  values of 25–500  $\mu\text{M}$  were reported. A high  $K_m$  value (55  $\mu\text{M}$ ) was also found for extracellular cAMP degradation in the frog muscle (Woo and Manery 1973). It is of interest in this connection that phosphodiesterase activity with high  $K_m$  values can also have an affinity for another cyclic nucleotide cGMP (Beavo *et al.* 1970, Russel *et al.* 1973).

For the prepubertal rat ovary the degradation of exogenously added cAMP to the incubation medium gave an apparent  $K_m$  value of 0.2  $\mu\text{M}$ . Phosphodiesterase activity with approximately the same  $K_m$  value as well as  $K_m$  values of 50–250  $\mu\text{M}$  have been found in homogenates of corpora lutea and prepubertal ovaries from the rat in studies from this laboratory (to be published). Stansfield *et al.* (1971) reported a high  $K_m$  value (250  $\mu\text{M}$ ) for homogenized bovine corpora lutea. The  $K_m$  values for the heart and the ovary demonstrated by the present investigation thus agree fairly well with those found in homogenates of these tissues. From the kinetic experiments in the present study it is obvious that the ovary degrades cAMP more rapidly than the heart at low extracellular concentrations of cAMP. This observation implies that the more pronounced accumulation of cAMP in the incubation media from the ovary after stimulation with LH (Selstam *et al.* 1974) than in the perfusate of the heart after stimulation with adrenaline (Isaksson, to be published) when the level of intracellular cAMP in the two different tissues is increased to approximately the same level cannot be explained by differences in degradation of exogenous cAMP between these tissues.

The degradation of cAMP from the medium might either be due to an uptake and subsequent degradation of cAMP intracellularly or to a degradation of cAMP by extracellularly active enzymes. For the ovary the degradation of cAMP was not inhibited when AMP or adenosine at a high concentration was added to the incubation medium although these substances almost completely inhibited the uptake of degradation products of cAMP, strongly suggesting that cAMP was degraded extracellularly. The possibility that the degradation of cAMP in the medium was caused by enzyme which had leaked out into the medium was ruled out in control experiments (see Results). The elimination of cAMP from the ovarian incubation medium thus seems to consist of a degradation of cAMP by phosphodiesterase which is attached to the cell surface. Similar observations have previously been reported by Woo and Manery (1973) using incubated frog skeletal muscle. For the heart it was not possible with the technique used to dilute effectively degradation products of cAMP by addition of AMP, since AMP was rapidly cleared from the perfusate. The question thus still remains whether the disappearance of cAMP from the heart perfusate is due to extracellular degradation of cAMP or to an uptake of cAMP with a subsequent intracellular degradation.

The degradation products of cAMP found in the ovarian incubation medium and heart perfusate included phosphorylated and dephosphorylated compounds. In the heart perfusate both  $\text{P}_i$  and adenosine were found indicating that AMP was further degraded to adenosine. The pattern of degradation seems to be the same in the ovary although the rate of AMP degradation proceeded more slowly than in the heart. In contrast to the pattern of degradation of AMP in the heart and the ovary found in the present study, AMP was

degraded to 5 inosine monophosphate (IMP) and inosine in frog skeletal muscle demonstrating that the pattern of AMP degradation differs between tissues and/or species (Dunkley *et al* 1966 Manery *et al* 1968) It cannot however be excluded from the present experiments that IMP and inosine might be intermediate metabolites in the degradation of AMP

The rate of disappearance of  $^3\text{H}$ -cAMP from the heart perfusate was approximately half the rate of  $^3\text{P}$ -cAMP disappearance The difference can probably be explained by a "recycling" of cAMP i.e. tritiated degradation products of  $^3\text{H}$ -cAMP were taken up by the myocardial cells and incorporated into ATP Tritium labelled cAMP is subsequently formed and released to the perfusate Since no detectable amounts of  $^3\text{H}$ -cAMP could be identified intracellularly the proposed release of  $^3\text{H}$ -cAMP must have been very rapid or cAMP must have been produced in close association to the cell membrane and then released The recycling hypothesis is supported by other observations demonstrating that adenosine is very rapidly taken up by the heart and incorporated into adenine nucleotides (Jacob and Berne 1960) In the ovary direct proof was obtained that adenosine was a degradation product of cAMP taken up by the cells since added adenosine but not adenine completely inhibited the uptake of  $^3\text{H}$  labelled substances

It is at present premature to more extensively speculate about the physiological importance of the degradation of extracellular cAMP Changes in the plasma levels of cAMP however accompany some endocrine diseases (for ref see Broadus *et al* 1971 Hardman *et al* 1971 Karlberg *et al* 1974) which might be due to changes in the activity of extracellularly active phosphodiesterase enzymes The question whether different hormones directly influence the activity of these enzyme(s) will therefore be of importance in forthcoming studies For one hormone insulin, evidence has been elicited that this hormone directly stimulates membrane bound phosphodiesterase enzymes (Woo and Manery 1973 Thompson *et al* 1973) It is however possible that membrane bound phosphodiesterase enzymes are most important for membrane and intracellular processes by having their substrate sites mainly towards the cell interior and that the degradation of extracellular substrates only reflects part of the characteristics of the enzyme A hypothetical function of extracellular cAMP might be that this nucleotide acts as an intercellular messenger within organs composed of many cell types such as the ovary The activity of membrane bound phosphodiesterase enzymes would consequently be of significance for this hypothetical function

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man and dog is indeed SP. To our knowledge this is the first demonstration that SP like material is present in blood.

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